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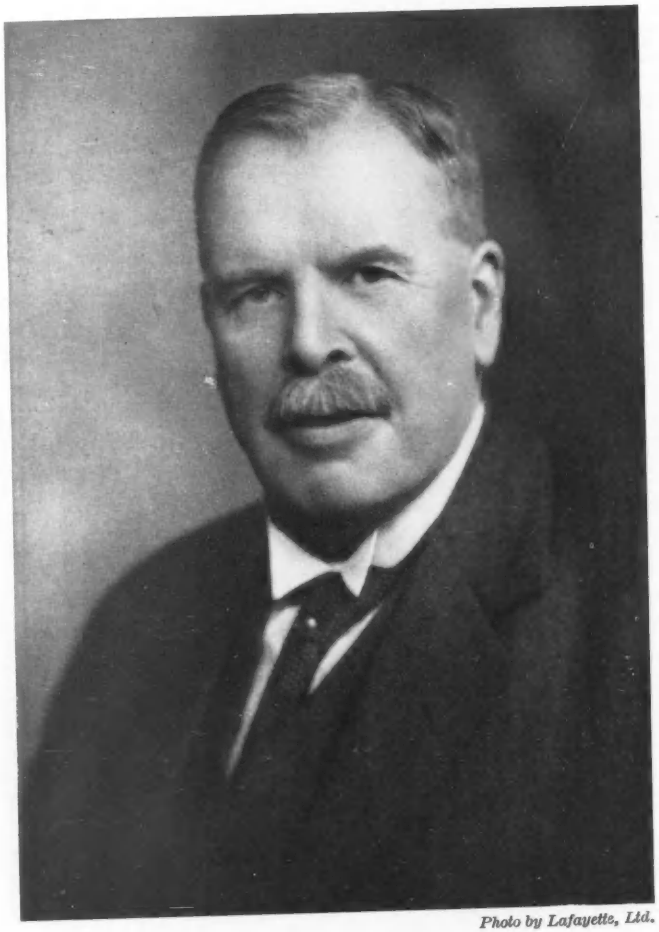

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J. A. GARDNER

Obituary Notice

JOHN ADDYMAN GARDNER, 1867–1946

John Addyman Gardner, who for many years past has been a familiar and beloved figure at nearly all Meetings of the Biochemical Society, passed away on 13 May 1946. The surviving original members of this Society will recall the part played by him in its foundation. Many will appreciate the long and able service he gave to the administration of the Society, and most of the present members will feel the loss of a kind and sympathetic friend.

Born in 1867 at Bradford, Gardner attended that city's Grammar School, now famous for its many distinguished pupils. Those qualities of kindness and geniality, of industry and enthusiasm, which have always been associated with Gardner, seem to have been prominent at Bradford. Maybe the Yorkshireman has something more than an average claim to these. It was not uncomplimentary on the part of his schoolfellows to have nicknamed him Bull Gardner. He appears to have excelled on the classical side, and it was not until after he had been elected to an open scholarship at Magdalen College, Oxford, in 1886, and after a period of residence there that he gave up his classical studies for chemistry, an example perhaps of his general ability and versatility. Amongst his fellow-students studying chemistry in the School of Natural Science, he soon became as popular as he had been at Bradford. Amongst his friends at Oxford there appeared many of the eminent chemists of his day. It was during his undergraduate days that he became attracted towards chemical research, an occupation which undoubtedly claimed by far the greater part of his activities during his lifetime. As an undergraduate he was for some time President of the Oxford University Junior Scientific Club, and was engaged upon other social activities amongst his scientific colleagues.

Gardner was awarded a Demyship while at Magdalen College and also the Daubeny Medal. In 1889 he graduated with first class honours in the School of Natural Science. Proceeding to Heidelberg, he completed some research under Victor Meyer, who, from a report now in the writer's possession, appears to have been yet another impressed with Gardner's ability to carry out research work "*mit grossem Fleiss und Eifer*". In 1891, Gardner returned to Oxford as Demonstrator in Chemistry. For three years, in collaboration with J. E. Marsh, he worked on the constitution of the terpenes (*J. chem. Soc.* 1891–94), a task of no mean order, since in those days the extraction of the required terpenes from suitable plants was often a necessary preliminary to such investigations.

In 1894 Gardner was appointed Lecturer in Chemistry and Toxicology at St George's Hospital Medical School, where he continued research on the terpenes, studying the bromination and oxidation products of camphene, camphoric and camphopyric acids. In those days the greater part of the hospital's clinical analyses and investigations were carried out under Gardner's supervision. The Medical School of St George's Hospital also provided pre-clinical courses for the London University Preliminary and Intermediate Examinations. Teaching and examining then occupied a large part of his time, and he became actively engaged on different Examination Boards. His frequent secondary school inspections were useful and popular. His interest in pre-clinical education caused him to take an active part in the introduction of a course of study and examination in organic chemistry in the London University 2nd M.B. To have secured such recognition of the growing importance of biochemistry was probably Gardner's first effort to advance this subject.

In 1905 Gardner was placed in charge of the chemical section of the Physiological Laboratory, which was attached to the London University side of the Imperial Institute at South Kensington, and known more generally as the Waller Laboratory, after its Founder and Director. Although retaining his position as Chemist to St George's Hospital, Gardner's interests were now directed more to South Kensington, where laboratory facilities for research were on a liberal scale. In these surroundings, Gardner, in collaboration with Buckmaster, undertook a laborious study on chloroform anaesthesia. An accurate method for the estimation of chloroform in blood was devised, and the anaesthetic and lethal quantities of chloroform were ascertained by a series of experiments on the cat. The rates of uptake and elimination and the function of the red corpuscles during anaesthesia were also investigated (*Proc. Roy. Soc.* 1906–7). It was at this time that Gardner began those researches on the sterols with which his name has become associated. Between 1908 and 1914, there appeared a number of communications under the title of 'The Origin and Destiny of Cholesterol in the Animal Organism'. The first with Dorée (*Proc. Roy. Soc.* 1908) referred to the discovery by Bondzynski that coprosterol, a reduction product of cholesterol, was excreted in human faeces, but

that the horse excreted hippocoprosterol. Pure coprosterol was prepared and a study of the faeces of the horse and other herbivora showed that hippocoprosterol was common to all, and that it originated from the grass on which the animals were fed.

Gardner and Dorée later showed that the cholesterol of the bile in the dog was reabsorbed, and that whether cholesterol or coprosterol was excreted depended largely on the nature of the diet. The coprosterol in faeces was shown to have its origin in the cholesterol of the diet. Coprosterol was not found in herbivora, and it was readily obtained from the faeces of a dog fed on a brain diet rich in cholesterol.

Gardner and Dorée prepared coprostenone and its derivatives, and by the action of sodium amyloxide obtained the epimer of coprosterol which they described as ψ-coprosterol. Work on the isomeric reduction product of cholesterol, referred to as β-cholestanol, was included. The action of ozone on cholesterol and cholestenone led to the opening up of one of the rings with the formation of an acid of lower carbon content.

With Ellis, Gardner showed that the cat excretes cholesterol and coprosterol, mostly the latter on a meat diet. With Fraser, he showed that the degree of inhibition of the haemolytic effects of saponin was due to the presence of cholesterol or phytosterol (as had been observed by Windaus), and followed the cholesterol content of the diets fed to animals. These authors found in the blood of rabbits fed on bran about 80 mg./100 ml. total cholesterol, of which only about one-sixth was in the form of esters. This amount varied slightly on diets free from or enriched with cholesterol. It was in this communication that Gardner and Fraser first utilized Windaus's discovery of the insoluble compound formed by cholesterol with digitonin; they investigated this compound in its application to cholesterol estimations and verified Windaus's observations on the accuracy attending its use in quantitative work.

Gardner commenced some experiments with a view to ascertaining how far cholesterol could be synthesized by the animal. He showed, with Ellis, that newly hatched chicks contained a little less, certainly not more, cholesterol, than could be accounted for in the original eggs. Some evidence that cholesterol was synthesized in adult subjects was obtained by Gardner and Fox (*Proc. Roy. Soc.* 1921) and subsequent work by other investigators has done much to remove any doubts that such synthesis takes place. In a series of experiments it was noted that a man, weight being constant, excreted slightly less coprosterol than could be accounted for by the cholesterol content of food intake. During an attack of influenza for the periods when the subject was losing weight, an excess of coprosterol was excreted, especially during a period when the loss of weight was heaviest (*Proc. Roy. Soc.* 1912).

At the same time Gardner continued to interest himself in chloroform anaesthesia. His attention was drawn to publications in which it was asserted that carbon monoxide was present in normal blood and especially in the blood of chloroform-anaesthetized animals. His previous elaborate researches with Buckmaster (quoted above) did not countenance these statements. By further careful and exact experiments with Buckmaster (*Proc. Roy. Soc.* 1909) he showed that the carbon monoxide, claimed to be found in the blood of anaesthetized animals, arose from the action of the caustic alkali on the chloroform content of the blood gases during carbon dioxide absorption; and the assertions that carbon monoxide was present in normal blood were also refuted.

Following this work Gardner and Buckmaster recorded some accurate observations on the venous and arterial blood gases of the normally breathing and of the anaesthetized cat. Working with hermetically sealed blood-gas pumps of the Töpler, Barcroft and Bone types, it is probable that some of the most accurate figures ever obtained in this type of analysis were made. Certainly, Gardner himself never undertook more laborious work (*J. Physiol.* 1910; *Proc. Roy. Soc.* 1910), in which one notes disagreement with the findings of the Chloroform Committee of the British Medical Association for 1910, to the effect that during chloroform narcosis the blood retains unimpaired its normal capacity of absorbing oxygen, and that the diminished concentration of this gas was solely due to slowing of respiration. According to Gardner and Buckmaster, diminished oxygen in anaesthetized blood was due to interference by the anaesthetic with the normal function of the red blood corpuscles. Accurate blood-gas analysis showed that the replacement of air by oxygen in respiration gave little increase of oxygen in the blood and no change in average carbon dioxide content.

Gardner was interested in the chemical constitution of cholesterol, and with Dorée (loc. cit.) he identified coprosterol as a secondary alcohol by its oxidation to coprostenone. With Williams (*Biochem. J.* 1913) he described further oxidation products of coprosterol and of the ketone. With Ellis (*Biochem. J.* 1918) he described some of the oxidation products of 'β-cholestanol' (cholestanol).

During the 1914–18 war, Gardner's previous acquaintance with the pungent lachrymatory substance chloropicrin led him to recommend its use in gas warfare, as an efficient but not too inhuman weapon. This volatile substance was produced in large quantities, under Gardner's supervision. During the same period he was responsible with Sir F. G. Hopkins for the comparison of the relative digestibilities of breads made from different flours.

Not content with these wartime responsibilities, he took an active share in the volunteer Police Force as a Special Constable.

Soon after the 1914–18 war the Physiological Laboratory at South Kensington closed.

Appointed Reader in Physiological Chemistry, he was lecturer in Organic Chemistry at the London School of Medicine for Women, but his major activities were still directed towards research, now largely confined to the Biochemical Laboratories at St George's Hospital Medical School. He was critical of methods described by different investigators for the determination of cholesterol and its esters. With Williams (*Biochem. J.* 1921) he showed that cholesterol esters developed colour with the Liebermann reagent at quite different rates compared with cholesterol, and never regarded the colorimetric method as accurate for total cholesterol. Some useful information concerning errors in gravimetric determinations of ester cholesterol, particularly those due to incomplete hydrolysis, was obtained in conjunction with Fox (*Biochem. J.* 1921, 1924).

Communications with Gainsborough (*Biochem. J.* 1927–38) dealt, for the most part, with cholesterol and its esters in plasmas; in these experiments are to be found some of the most accurate determinations recorded for the cholesterol and cholesterol ester contents of normal plasma together with the limit of variations on 50 subjects examined. Sources of error in such determinations were ascertained, and errors due to the difficulty of separating sterols from protein matter were also recorded. With Murray, Gardner and Gainsborough noted an error due to shrinkage of the corpuscles by the addition of certain anti-coagulants, with as much as a 4 % increase in the plasma volume.

Gardner and Gainsborough drew attention to the large amount of cholesterol found in the total blood of a man, as compared with the amount to be found in an average daily diet. The whole of the latter, if taken into the blood stream, might effect an increase within the normal variations of the sterol content.

During this period (*Brit. med. J.*, *Lancet*, *Quart. J. Med.* 1930–38) Gardner and Gainsborough were also engaged in studying the sterol content of plasma under different normal and pathological conditions. Hypercholesterolaemia was not produced by a simple cholesterol-rich meal, though it would appear on continuing such diets when there was a greater increase of cholesterol ester than of cholesterol in the plasma. So also could hypocholesterolaemia be occasioned on a prolonged cholesterol-low diet, but this resulted in a lowering of ester and not of free cholesterol. The influence of a simple diet on the plasma cholesterol is more marked by variations in the type of food than by variation in cholesterol content. This may result in an increase or decrease in the plasma content, and these variations are regarded as due to endogenous metabolic changes in which cholesterol plays a part. Further work demonstrated the close attachment of cholesterol to the euglobulin fraction of plasma proteins, the presence in normal urine of minute quantities of cholesterol which were considerably increased in some forms of nephritis, and the reversal of the normal ratio of ester to free cholesterol in the plasma during pregnancy. The normal ratio of approximately 2 : 1 became inverted between the 24th and 32nd week of pregnancy but returned to normal before termination; at the same time the hypercholesterolaemia noted by other observers was a less constant finding. Studies were also made on nephritis, hyperthyroidism, myxoedema and on hepatic and biliary disease. In the latter it was demonstrated that partial or complete biliary obstruction was followed by a marked lowering of the ester cholesterol of the plasma, but that later if obstruction was complete hypercholesterolaemia ensued with, however, a reversal of the ester to free cholesterol ratio. In hepatic disease the plasma cholesterol changes were dependent on the flow of bile to the intestine. The changes in the amount of plasma cholesterol in ester form were presumably related to the effect of biliary stasis on the absorption of fat and cholesterol from the intestine.

Reverting to the investigation of faeces, Gardner and Gainsborough analyzed the intestinal contents obtained from a patient with a terminal ileostomy and found that, while there was practically no coprosterol present, the quantity of β-cholestanol was very much higher than in colonic faeces.

During the latter part of this period of work Gardner was handicapped by illness. Although diabetes was well controlled, other affections reduced his capacity, and much other work on faecal sterols, the sterols of fungi, and the role of deoxycholic acid was never completed.

Gardner's interests were wide and varied. While at St George's Hospital, his clinical examinations extended over a period of more than 50 years. He wrote and lectured on the sulphanilamide drugs. With Buckmaster he contributed a comprehensive account of 'Blood' to Allen's *Commercial Organic Analysis*, in which his love of accuracy is to be found in the careful and minute description of the methods for determining spectrographic absorption. Perhaps his only pastime was fishing in the Yorkshire rivers, and perhaps it was for this reason that Gardner was always interested in any investigation concerning the pollution of river waters. He was a member of the Scientific Advisory Committee set up in 1924 to advise the Standing Committee on River Pollution under the Ministry of Agriculture and Fisheries. His considerable experience in the accurate determinations of blood gases led him to make use of his tapless and jointless modification of

the Töpler pump for the difficult investigation of the oxygen consumption of different fresh-water fish. The relatively small gas volume relative to water volume demanded a high degree of accuracy and manipulative skill. Working with Leathes and later with Powers and King (*Biochem. J.* 1914, 1922, 1923) he found that the oxygen consumption of fish approximately doubled for a rise of 10°, and that below 10° the respiratory quotient fell appreciably, suggesting a fundamental change of metabolism. Of the different fish experimented with, trout were asphyxiated by a reduction of normal oxygen tension to 1/3, gold fish would require a reduction to 1/10, and eels still less.

Gardner was one of the founders of the Biochemical Society. At a meeting called at University College, London, in January 1911 Gardner acted as Chairman and explained the reasons for the formation of a Society which should include the many and wide interests of Biochemistry. The audience was very interested and proposed immediate action. Rules were drawn up and the first meeting was held in March 1911. At the subsequent dinner the rules were passed. After discussion it was deemed preferable to call the association The Biochemical Club, mainly for the reason that it did not possess a *Journal*. At that time the *Biochemical Journal* was owned and edited by the late Professor Benjamin Moore and Dr Whitley and published in Liverpool. Professor Moore became a member of the Biochemical Club. Negotiations were initiated for the publication of a *Journal* and ultimately it was arranged with Moore and Whitley that the *Biochemical Journal* should be purchased by the Biochemical Club. Gardner was active in these negotiations. The purchase was completed early in 1913. At the Annual Meeting in March of that year the association became the Biochemical Society. Plimmer was appointed Hon. Secretary and Gardner Hon. Treasurer. The membership of the Society was then under 200, and during the war years, 1914–18, Gardner managed the financing of the *Journal* so that the Society was never in debt. Though the work of the Treasurer increased year by year Gardner was reluctant to relinquish his duties and remained Treasurer for over 30 years. Thanks to his careful management he saved a considerable surplus of funds which were placed in the hands of Trustees. Gardner acted throughout as convenor and secretary of the Trustees. The present firm financial foundation of the Biochemical Society is entirely due to Gardner.

In 1903, Gardner married Mrs Henrietta White, who died in 1926. This charming and highly accomplished lady was hostess at many pleasant gatherings in their Kensington house. During the 1914–18 war Mrs Gardner was appointed by the War Office as interpreter for the lesser known Nordic languages. During the last war Gardner was an active Warden. Cool under all emergency, his presence in the neighbourhood of High Street, Kensington, during all heavy raids was invaluable.

On retirement from St George's Hospital in 1942 with no abatement of his enthusiasm for research, he continued to work in the writer's laboratory at St Mary's Hospital Medical School. While so engaged he received by telephone during the V-rocket period, the message that his sister, Miss Gardner, had been injured and their flat destroyed. This seemed to forecast the cessation of Gardner's work. Fortunately, Miss Gardner was not seriously hurt. Failing to find accommodation in London, they left for Bradford, hoping to return later.

Gardner was a connoisseur of good food and good wine. He was also a connoisseur of good men, never happier than when in their company. Full of kindliness towards his fellow-men, he was hurt by the absence of such in others. A beloved member of the Old Bradfordians, a Knight of the Round Table, and a member of a number of Masonic Lodges in London and at Bradford, he was the friend of all. His punctilious sense of responsibility, and no doubt his desire not to lose touch with so many friends, brought him back to London in all weathers to preside at his Lodge functions. He died at Bradford from heart failure following bronchopneumonia. Since the death of Mrs Gardner he had been under the constant care of his sister, Miss Mary Gardner, whose devotion to her brother is well known to many members of the Biochemical Society.

G. W. ELLIS

BAL-INTRAV: A New Non-toxic Thiol for Intravenous Injection in Arsenical Poisoning*

By J. F. DANIELLI, MARY DANIELLI, (the late) J. B. FRASER, P. D. MITCHELL, L. N. OWEN and G. SHAW, *The Biochemical Laboratory, Cambridge, and the Department of Organic Chemistry, Imperial College of Science and Technology, London, S.W. 7*

(*Received* 2 *November* 1946)

1. BIOLOGICAL OBSERVATIONS

By J. F. DANIELLI, MARY DANIELLI and P. D. MITCHELL

This paper is based on a report to the Ministry of Supply made in March 1944, and describes investigations culminating in the production of BAL-INTRAV (2:3-dimercaptopropanol glucoside), a non-toxic thiol, affording a high degree of protection against lewisite poisoning, and likely to be of value in other forms of arsenical, metal and metalloid poisoning.

A series of fundamental investigations in the Department of Biochemistry at Oxford (Peters, Stocken & Thompson, 1945), based on the view that the lethal action of lewisite is caused by its combination with chemical groups in enzymes, particularly SH groups, has resulted in the production of BAL (2:3-dimercaptopropanol). This substance, when applied to the skin of a contaminated animal, provides a considerable measure of protection against the vesicant action of lewisite. But as an antidote to systemic poisoning, BAL suffers from the disadvantage of rather high toxicity, and so must be used in relatively small amounts fairly soon after contamination, and cannot be used for intravascular injection in large amounts. The LD_{50} of BAL, when given to rabbits by intramuscular injection, is about 50 mg./kg., i.e. about 10 times the LD_{50} (by weight) of lewisite.

Meanwhile, studies at Porton by Cameron and his colleagues (1940, 1941) had shown that in lewisite poisoning there is, in addition to the local lesion on the skin, a condition of shock with marked haemoconcentration, and a pronounced systemic effect involving damage especially to the liver and lungs. Serum therapy and other anti-shock measures were shown to be useless as a means of prolonging life, and it was concluded that until the arsenical could be removed from the body, or in some way inactivated, little could be done by way of therapy. Further studies at Cambridge showed (*a*) that perfusion of lewisite increases the permeability to protein of the capillaries of the frog (Danielli & Danielli, 1941*a*) and that this will occur with a dosage of as little as 0·2 mg./kg. (Danielli & Danielli, 1941*b*); (*b*) that an increase in rate of loss of plasma appears prior to any apparent haemo-concentration (Cameron, Short, Mitchell & Danielli, 1943); (*c*) that with a dosage of 4 mg. lewisite/kg. (*c.* 1 LD_{50}) given to 2 kg. rabbits, the rate of loss of protein from the blood stream has become marked after 1 hr. and almost maximal 6 hr. after contamination, and (*d*) that of the increased rate of protein loss, 95 % was systemic and only 5 % was due to the local lesion (Mitchell, Danielli & Short, 1944).

The conclusion was drawn that therapeutic measures must involve two effects; first, the inactivation of the arsenical and preferably its removal from all sites, including intracellular sites, which it has reached, and second, recuperation of the tissues from the abnormalities produced by the arsenical invasion. To achieve the first part of this programme a drug is required with the following properties:

(1) It shall be so non-toxic that no danger exists of complications from an overdose, even when given intravenously. (2) It shall prevent arsenicals penetrating from the blood stream into cells. (3) It shall remove arsenicals from the cells into which they have penetrated. (4) The reaction product between the drug and the arsenical shall be readily excreted.

To meet requirements (1), (2) and (4), use may be made of the principles involved in the normal detoxication processes carried out by the living organism itself, discussed elsewhere (Danielli, 1942).

Use of inability to penetrate the cell membrane is seen in detoxication mechanisms, in which toxic substances are conjugated with amino-acids, with sulphuric acid or with glucuronic acid, so that a toxic, cell-penetrating substance such as bromobenzene or menthol is converted into a new molecule which cannot penetrate into cells, and which, once reaching the blood stream, is bound to be filtered off by the glomerulus of the kidney, and will not be resorbed from the urine in the tubules unless it happens to fit into

* A preliminary account of this work has been given elsewhere [*Nature, Lond.*, **157**, 217 (1946)].

the rather specific secretory mechanism of the tubules. Thus by conjugation with a molecule such as glucuronic acid, two objects are secured simultaneously: (*a*) the toxic substance is prevented from penetrating into cells and (*b*) its excretion into the urine is secured.

Thus, we expected that the toxicity of BAL would be greatly diminished on conversion into its glucuronide or ethereal sulphate, since the ability of the substance to penetrate into cells would then be much reduced. It would at the same time circulate freely in the blood stream, intercellular spaces and lymphatics and would sweep these spaces clear of arsenical since its affinity for arsenic should be almost unchanged; it would also be readily excreted in the urine. From the point of view of synthesis, BAL glucuronide and ethereal sulphate have proved difficult of access, but the required physico-chemical properties connected with permeability may be obtained by use of any molecule containing a number of hydroxyl or carboxyl groups (Danielli, 1943). We have therefore investigated such molecules as BAL glucoside, mercaptosuccinic, and dimercaptoadipic acid.

So far we have dealt with points (1), (2) and (4) above. The remaining point, (3), concerns the removal of arsenic from cells into which it has already penetrated. It might be thought that if the thiol is incapable of penetrating cells, so its ability to remove arsenic from them will be destroyed. This is not so. Most cells contain sufficient reduced glutathione to prevent arsenic being completely localized at a particular intracellular site or becoming completely indiffusible: there must be an equilibrium between the indiffusible arsenic fixed by SH and other groups of proteins, and the diffusible arsenic associated with SH groups of glutathione. In the course of this work we have obtained evidence that glutathione may pass relatively rapidly through some cell membranes. Consequently we may expect that arsenicals will not be completely inaccessible to a thiol drug, even if the drug is completely unable to penetrate into cells, since by virtue of the above equilibrium the arsenic will be slowly removed by the thiol. Thus we anticipated that the compounds conforming to requirements (1), (2) and (4) would also fulfil requirement (3).

EXPERIMENTAL

Treatment of animals. Unless otherwise stated, rabbits of 1·5–2·5 kg. in weight were used. Lewisite (12 mg./kg.) was applied to an area of the back from which hair had been clipped. Subsequently, the animals were given unrestricted access to dry food, green food and water. Sick animals were kept in a warm room until recovery or death.

Preliminary experiments showed that the LD_{50} for the sample of lewisite used, and for the size of rabbit used, was *c.* 4 mg./kg. or less. In choosing the dosage of lewisite to be used we were influenced both by shortage of rabbits and of technical assistance, which jointly precluded the possibility of using large numbers of animals. It was therefore impossible to study small effects which could only be established by statistical analysis. We therefore used a large dosage of lewisite (12 mg./kg.) which killed all animals with certainty in 10–20 hr. if no therapeutic measures were taken. If, after therapy, there ensued marked prolongation of life or survival, we could be quite sure these results were significant and indicated effective therapy.

Immediately before injection the batch of thiol was neutralized to thymol blue, using concentrated HCl or NaOH, and then diluted to be approximately isotonic with plasma. We endeavoured to give a dose of thiol of at least 20 equivalents of the amount of lewisite applied.

No precautions of any kind were observed with regard to sterility.

RESULTS

Toxicity tests

Before use, toxicity tests were made to ensure that the dose of thiol to be used for therapy was not in itself toxic. The rabbits employed for these experiments were given at least twice as much thiol as was to be used for therapy. The dose was split into 5 parts, given intravenously at intervals of 1 hr. Blood samples were taken at intervals over 24 hr. after the first injection and haematocrits determined. Approximate values of LD_{50} for various thiols are given in Table 1. Since only two animals were studied for a given dosage, these are only rough values and should be regarded as significant only as regards order of magnitude. In no case was significant haemo-concentration observed following administration of the thiol.

Table 1. *Toxicity of various thiols*

(2 kg. rabbits; thiol given intravenously*)

Thiol	Approx. LD_{50}/kg. (mg.)
BAL (dimercaptopropanol)	50
Mercaptoacetate	100
Mercaptosuccinate	>1000
Mercaptoadipate	>1000
Acetylmercaptosuccinate	>1000
αα′-Dimercaptoadipate	>1000
Glutathione	>2000
Glucothiose	1000
BAL glucoside (BAL-INTRAV)	>5000

* See Part 2 of this paper for details of synthesis.

The Table shows that the toxicities fall in the order BAL > mercaptoacetate > glucothiose > mercaptosuccinate, mercaptoadipate, acetylmercaptosuccinate, dimercaptoadipate, glutathione and BAL glucoside (BAL-INTRAV). Drs Thompson and Stocken have informed us that trithioglycerol is more toxic than BAL. Thus the toxicities fall into the order required by the theory given above, with the possible exception of glucothiose. With fatal doses of BAL and mercaptoacetate, death occurs

fairly soon after intravenous injection, whereas with glucothiose, only after 4–5 days. We therefore suspect that the former two compounds penetrate rapidly into cells and may cause death by one mechanism, whereas glucothiose penetrates slowly and causes death by a different mechanism, possibly by competition with glucose for some enzyme centres.

Whilst conducting the experiments reported here with mercaptosuccinate, acetylmercaptosuccinate, mercaptoadipate, dimercaptoadipate, glutathione and BAL-INTRAV, we had insufficient material to determine the LD_{50} or even to observe marked symptoms of toxicity. To rabbits, the toxicity of these compounds is so low as to be of no practical importance. Larger supplies of BAL-INTRAV became available later, and a report on its toxicity will be given in another paper (Danielli, Jones & Mitchell, in preparation).

As indicated by the nitroprusside test, mercaptosuccinate and BAL-INTRAV persist in the blood stream for some hours after injection. On the other hand, when 3·6 g. of reduced glutathione were injected intravenously into a 3 kg. rabbit, at least 95% of the glutathione had vanished after 8 min. from the blood stream. This loss was not produced by oxidation to GSSG, or to entry of glutathione into red blood cells. There must be some cellular system in the body which removes glutathione from the blood stream with great rapidity.

Relative therapeutic efficiency of different thiols

Two rabbits were used with each thiol; they were painted with 12 mg. lewisite/kg. Intravenous and subcutaneous injections of thiol were given at 0, 1, 2, 4 and 6 hr. after contamination with lewisite. About half the dose was given by injection into the marginal ear vein, and the remainder by subcutaneous injection made in the vicinity of the contaminated area.

The results are shown in Table 2. Many of the thiols were able to prolong life, and in borderline cases (i.e. with less heavy contamination with lewisite) might perhaps save life. But only the dithiols (dimercaptoadipic acid and BAL-INTRAV) had a very pronounced effect, and of these two compounds, BAL-INTRAV alone prevented death with the dosage used. We therefore made a more intensive study of BAL-INTRAV.

The conditions under which BAL-INTRAV will ensure survival

In their studies Cameron and his colleagues found that profound damage to the liver occurs at an early stage in lewisite poisoning. In our studies on lewisite shock (Mitchell, Danielli & Short, 1944) we found that with a dose of only 4 mg. lewisite/kg., systemic damage to capillary function is significant after 1 hr. and almost maximal after 6 hr. These changes must occur even more rapidly with the dosage used in the present experiments (12 mg./kg.). It is therefore clear that damage to important cellular systems commences at an early stage, and that if this progresses too far, removal of the arsenic by administration of BAL-INTRAV cannot prevent death. We have therefore made an attempt to find how long the administration of BAL-INTRAV may be delayed. The results are given in Table 3. As before, the BAL-INTRAV was given in

Table 3. *The effect of administration of a total of 1·5 g. BAL-INTRAV/kg. at various times after contamination of 2 kg. rabbits with 12 mg. lewisite/kg.*

Time after contamination before first injection of BAL-INTRAV (hr.)	No. of animals	Survival time after dosage (hr.)	Survivors (%)
0	2	None died	100
2	7	,,	100
4	2	,,	100
6·5	8	,,	100
12	4	12, 12, 13, 13	0

Table 2. *Effect of different thiols on rabbits treated with lewisite*

(2 kg. rabbits; 12 mg. lewisite/kg. painted on the skin. Injection of thiol was commenced immediately after contamination.)

Thiol	Dosage (g./kg.)	No. of animals	Survival time after dosage (hr.)	Survivors (%)
None	—	11	10, 12, 15, 18, 13, 13, 14, 13, 14, 14, 15	0
Glutathione	0·7	2	12, 18	0
BAL	0·07	2	10, 15	0
Mercaptosuccinate	0·5	2	48, 70	0
Acetylmercaptosuccinate	0·75	2	20, 40	0
Mercaptoadipate	0·5	2	43, 65	0
Glucothiose	0·5	2	24, 26	0
Dimercaptoadipate	0·75	2	72, 260	0
BAL-INTRAV	1·5	2	None died	100

4–5 doses, half intravenous and half subcutaneous by rapid hypodermic injection.

Animals treated up to 4 hr. after contamination had a moderate lesion on the back, but made an uneventful recovery without loss of appetite at any stage. Animals treated 6·5 hr. after contamination also recovered completely, but were rather ill and of poor appetite for 1–3 days. But animals treated 12 hr. after contamination (i.e. on the point of death) did not appear to be in any way assisted by this late injection of BAL-INTRAV.

We think it probable that after 12 hr. so much cellular damage has been done by this heavy dosage with lewisite that irreversible processes have set in. Our present knowledge of cell physiology is so slight that there can at present be little hope of developing a method of effective therapy once damage has progressed to such a stage that removal of arsenic is inadequate.

Complications and checks

A number of further factors needed checking. These were: (*a*) The reproducibility of therapeutically effective BAL-INTRAV. The possibility existed that repetition of the synthesis might prove impossible. This possibility has been eliminated: the results of Table 3 were obtained with two different batches of BAL-INTRAV, and numerous further batches have since been examined (Danielli, Jones & Mitchell, in preparation). No difference in the effectiveness of the batches was observed.

(*b*) Deterioration on storage might be serious. We have not studied this in detail, but one sample appeared to have unimpaired activity after storage for 3 months at 2°.

(*c*) Different batches of lewisite are of different toxicity. The sample used in the experiments described above appeared to be more toxic than most. Four experiments (with treatment of two rabbits 4 hr. after contamination and of the other two 6·5 hr. after contamination) showed that BAL-INTRAV coped just as readily with a sample of lewisite which (at the same dosage of 12 mg./kg.) produced death in about 24 hr.

(*d*) The size of the animal is likely to be an important variable. For example, young rabbits have very thin skins and so may absorb lewisite more rapidly than older animals; they are also usually less resistant to experimental procedures. We therefore carried out a number of experiments with young rabbits of about 600 g. weight. The results are recorded in Table 4. It is clear that young animals must be treated sooner after contamination than is essential with older animals.

Table 4. *The effect of administration of* 1·5 *g./kg. of BAL-INTRAV to young* (600 *g.*) *rabbits at various times after contamination with* 12 *mg. lewisite/kg.*

Time after contamination before first injection of BAL-INTRAV (hr.)	No. of animals	Survival time after dosage (hr.)	Survivors (%)
4	4	None died	100
6·5	4	14, 12, 12, 80	0

2. CHEMICAL OBSERVATIONS

By J. B. FRASER, L. N. OWEN and G. SHAW

For the reasons already mentioned above, the chemical portion of the investigation was directed towards the preparation of water-soluble thiols containing several carboxyl or hydroxyl groups, and the first experiments were concerned with the former type of compound.

Very few dibasic mercapto-acids have been described in the literature. Mercaptosuccinic acid, and mercaptomethylsuccinic acid are most conveniently prepared by reaction of thioacetic acid (CH_3COSH) with maleic and itaconic acids respectively, followed by removal of the acetyl group by mild alkaline hydrolysis (Holmberg & Schjanberg, 1940). Similarly, by the action of thioacetic acid on allylmalonic acid, Schjanberg (1941) has prepared γ-mercaptopropylmalonic acid. An older method in which the halogenated acid is treated with potassium thioacetate and the resulting compound hydrolyzed to the mercapto-acid was applied by Fredga (1938) to the synthesis of αα′-dimercaptoadipic acid from αα′-dibromoadipic acid.

It has now been found that, as would be anticipated, thioacetic acid reacts readily with αβ-unsaturated esters. With methyl acrylate it gave methyl β-*S*-acetylmercaptopropionate, from which β-mercaptopropionic acid was obtained on saponification, and with ethyl maleate it yielded ethyl *S*-acetylmercaptosuccinate, which on saponification gave mercaptosuccinic acid.

The reaction between potassium thioacetate and halogeno-compounds, to give acetylthiols, which had hitherto been used only for the preparation of neutral thiols (Gehrke & Kohler, 1931; Sjoberg, 1942; D. R.-P., 557, 247), has now been applied to the formation of mercapto-acids through the esters. The reaction proceeds in ethanolic solution and is therefore particularly useful for the treatment of halogeno-esters, which, being practically insoluble in water, do not readily condense with potassium ethyl xanthate. Thus from ethyl α-bromopropionate, ethyl α-*S*-acetylmercaptopropionate was prepared, which on saponification gave α-mercaptopropionic acid. Similarly, ethyl α-bromoglutarate was converted into ethyl α-*S*-acetylmercapto-

glutarate and thence into α-mercaptoglutaric acid; ethyl α-bromoadipate was used for the preparation of α-mercaptoadipic acid. It was hoped to prepare αα′-dimercaptosuccinic acid, but efforts to bring about the addition of thioacetic acid to chloromaleic acid resulted only in the formation of chlorofumaric acid, and although a reaction occurred on treatment of ethyl αα′-dibromosuccinate with potassium thioacetate, the product obtained on saponification contained no sulphur.

In the carbohydrate field, monothiols derived from several sugars have been described. In general, these have been of the glucothiose type, prepared from the acetohalogeno-sugar *via* the disulphide, xanthate, or acetylthioderivative (Wrede, 1922; Schneider, Gille & Eisfeld, 1928; Gehrke & Kohler, 1931; D. R.-P., 557, 247); an exception is the 6-thioglucose of Ohle & Mertens (1935) prepared from a 5:6-anhydro-sugar. Mercaptoalkylglucosides, however, are unknown. Unsuccessful attempts were made to condense acetobromoglucose and BAL, and it was decided to attempt the preparation of BAL glucoside by the following route: Acetobromoglucose → tetra-acetyl β-allylglucoside → tetra-acetyl β-(2′:3′-dibromopropyl) glucoside → hexa-acetyl BAL glucoside → BAL glucoside. The dibromide, previously described by Fischer (1920), reacted rapidly with potassium thioacetate in boiling ethanol, and the syrupy product was deacetylated by the Zemplen catalytic procedure with slightly more than two equivalents of barium methoxide in methanol to yield a barium salt of the thiol. Biological tests on a solution of the free glucoside, obtained by removal of barium as sulphate, were highly satisfactory, and no difficulty was experienced in repeating the operations in the synthesis, but for large-scale work it was obviously desirable to devise an alternative procedure which would avoid the necessity of using acetobromoglucose.

The direct formation of glycosides by the Fischer method (condensation of sugar and alcohol in the presence of hydrogen chloride) proceeds smoothly and in good yield with the lower aliphatic saturated alcohols, in which the sugar is appreciably soluble, but no success was achieved in attempted condensations of glucose with 2:3-dibromopropanol. The method, however (cf. Fischer, 1893), has now been found to be applicable to the preparation of allylglucoside. This section of the work was completed in 1944. More recently, Talley, Vale & Yanovsky (1945) have reported the preparation of the same substance by a similar process, though the yield was inferior to that obtained by the method now to be described. Glucose readily passed into solution when warmed with allyl alcohol containing 3 % of dry HCl; but on continued heating the HCl concentration gradually diminished, owing to the formation of allyl chloride, and for optimal yields it was found necessary to pass in more dry HCl from time to time. After neutralization and evaporation, the mixed α- and β-allylglucosides were, after extraction with boiling acetone, obtained as a syrup which slowly crystallized. Attempts were made to brominate this material in dioxan solution, but the product was very unstable, and the glucosides were therefore acetylated prior to bromination. A portion of the acetylated product, purified by chromatography, was separated by fractional crystallization into tetra-acetyl-α-allylglucoside, and the already known β-isomer. For general purposes, this tedious separation was unnecessary, since there was no reason to suppose that a mixture of the α- and β-glucosides of BAL would be inferior to the β modification originally prepared. The main bulk of the crude tetra-acetate was therefore brominated, treated with potassium thioacetate, and saponified, according to the original procedure. This new specimen of BAL-INTRAV was found to be no less satisfactory than that prepared by the older route. An attempt was also made to convert the dibromide directly into BAL-INTRAV by treatment with sodium hydrosulphide, but the difficulty of separating the product from excess reagent and inorganic salts rendered the modification impractible.

BAL-INTRAV has not so far been prepared in a chemically pure state.* The free dithiol (prepared by neutralization of the sodium or barium salt) resinifies very readily with almost complete loss of thiol reaction when its solutions are evaporated. Chromatographic separation was unsuccessful, probably owing to the insolubility of the compound in organic solvents other than alcohols and dioxan. The mercury salt decomposes rapidly when dried *in vacuo*, and both the lead and silver salts are very soluble in water, and consequently difficult to isolate. In this connexion it is of interest that AgCl is readily soluble in BAL-INTRAV solution, a behaviour which also occurs with many other mono- and dithiols; this is undoubtedly due to the great affinity of thiol groups for the ions of heavy metals. Analyses of the barium and sodium salts of BAL-INTRAV have always given anomalous results, the thiol content, determined by titration with iodine, being less than the total sulphur (see Table 5), but unlike BAL, which on titration with iodine gives a sharp end-point, BAL-INTRAV continues slowly to take up iodine. These results have also been confirmed by American workers (N.D.R.C. Report, 1945). It is possible that crude BAL-INTRAV is present partly in an anhydro-form, derived by loss of water between one of the thiol groups and a hydroxyl group in the glucose moiety, comparable perhaps with the anhydro-glycolglucosides of Helferich & Werner (1942; 1943); in this connexion it may be significant that dithioglycide (anhydro-BAL) reacts with iodine in a similar manner, taking up one equivalent immediately and a further quantity much more slowly. Alternatively the low sulphur

* Mr R. M. Evans, of the Imperial College Laboratories, has recently succeeded in obtaining the hexa-acetate of BAL glucoside in pure crystalline form. Details of this work will be published shortly.

value may be explained by partial formation of a thioanhydro ring by loss of hydrogen sulphide. In either event, the metal content should also be low, and this is indeed observed for the sodium salt, but is not so marked in the case of the barium salt. The higher metal content in the latter case may be due to co-precipitation of barium acetate with barium BAL-INTRAV; this difficulty would not be expected to arise to the same extent with the sodium salt, owing to the much greater solubility of sodium acetate in ethanol.

Table 5. *Analysis of salts of BAL-INTRAV*

	Metal (%)	Sulphur: Total S (%)	Sulphur: Thiol-S (%)
Sodium salt (i)	6·9	16·9	8·4
(ii)	7·6	13·9	9·2
(iii)	—	11·9	8·0
$C_9H_{16}O_6Na_2S_2$ requires	13·9	19·4	19·4
Barium salt (i)	33·4	16·8	9·8
(ii)	27·0	8·1	5·7
(iii)	30·4	12·5	7·7
$C_9H_{16}O_6BaS_2$ requires	32·6	15·2	15·2

It was thought that polarographic analysis might be capable of detecting a 1:2-dithiol, and thereby assist in confirming the structure of BAL-INTRAV. Brdicka (1933) reported that very small amounts of cysteine can be estimated polarographically in the presence of the cobaltous hexammine ion. The curves he obtained showed a maximum at $-1{\cdot}2$ to $-1{\cdot}3$ V. due to the cobaltous ion; and also a second maximum at $-1{\cdot}8$ V. characteristic of the thiol group; the height of the latter above the cobaltous maximum being dependent, within certain limits, on the concentration of thiol in solution. This wave maximum at $-1{\cdot}8$ V. was given by mercaptoacetic acid, but not by glutathione.

In order to determine whether this effect was general for substances containing free thiol groups, and in particular if it could be used to distinguish between the two thiol groups in BAL and its analogues, several representative thiols were submitted to polarographic examination. In each case, a varying concentration of thiol was used in M-NH_4OH/NH_4Cl buffer solution (pH 9·8) containing $CoCl_2$ at a concentration of 10^{-3} M. Measurements were made against a standard calomel electrode with a Cambridge Voltamoscope.

All the α-mercapto-acids examined showed thiol peaks at approximately $-1{\cdot}8$ to $-1{\cdot}9$ V., although the heights of these waves were different at any particular concentration; the high value for αα′-dimercaptoadipic acid is especially noteworthy. Each acid was examined over a wide range of concentrations, but, for simplicity, only the curves for a concentration of $2{\cdot}5 \times 10^{-4}$ M are shown in Fig. 1.

β-Mercapto-acids gave very different results. β-Mercaptopropionic and mercaptomethylsuccinic acids showed only a slight inflexion at *c.* $-1{\cdot}8$ V., there being no characteristic peak similar to that observed for α-mercapto-acids. For comparison,

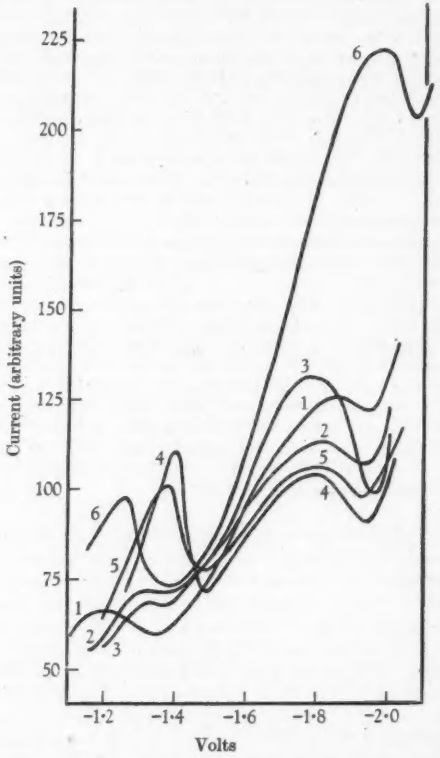

Fig. 1. Polarograms of α-mercapto-acids. 1, Mercaptoacetic acid. 2, α-Mercaptopropionic acid. 3, Mercaptosuccinic acid. 4, α-Mercaptoglutaric acid. 5, α-Mercaptoadipic acid. 6, αα′-Dimercaptoadipic acid.

cysteine was examined at very low concentration, and gave the expected peak at $-1{\cdot}8$ V. (Fig. 2). It has been reported that homocysteine, a γ-mercapto-α-amino acid, gives curves almost identical with those of cysteine, and it appears, therefore, that the presence of the amino group greatly increases the sensitivity of the cobalt-catalyzed polarographic reaction of mercapto-acids.

No thiol inflexion whatsoever was given by BAL, though it did have a marked influence on the general shape of the curve. With thioethyleneglycol, some

indication of an inflexion was observed (Fig. 3), but it is evident that in the absence of a carboxyl group

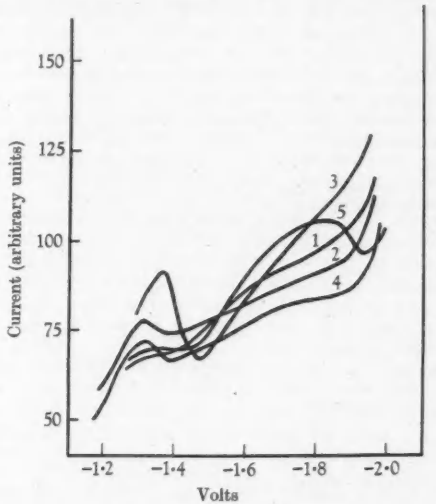

Fig. 2. Polarograms of β-mercapto-acids. 1, β-Mercaptopropionic acid, 10^{-3}M. 2, β-Mercaptopropionic acid, $2{\cdot}5 \times 10^{-4}$M. 3, Mercaptomethylsuccinic acid, 10^{-3}M. 4, Mercaptomethylsuccinic acid, $2{\cdot}3 \times 10^{-4}$M. 5, Cysteine, 10^{-5}M.

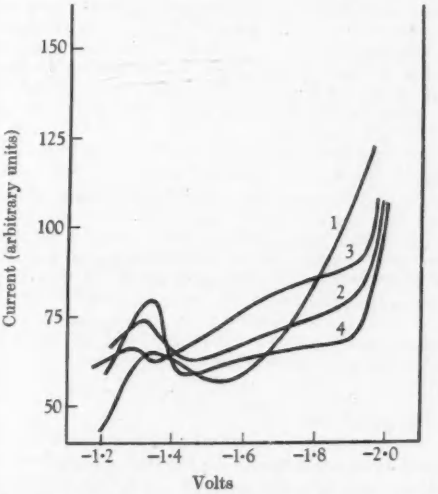

Fig. 3. Polarograms of mercapto-alcohols. 1, BAL, 10^{-4}M. 2, BAL, 5×10^{-6}M. 3, Thioethylene glycol, 10^{-3}M. 4, Thioethylene glycol, $2{\cdot}5 \times 10^{-4}$M.

the polarographic detection of thiols is not possible with the methods at present available.

EXPERIMENTAL

S-Acetylmercaptosuccinic acid, mercaptosuccinic acid, and mercaptomethylsuccinic acid. These were prepared by the method of Holmberg & Schjanberg (1940) and had m.p. 123–124°, 151°, and 106°, respectively. αα′-Dimercaptoadipic acid, m.p. 183°, was obtained by the procedure of Fredga (1938).

Glucothiose. Thioacetic acid (12 g.) in ethanol (50 ml.) was neutralized with 3N-ethanolic KOH and added to a solution of acetobromoglucose (55 g.) in warm ethanol (100 ml.). There was an immediate precipitation of KBr, and after 6 hr. at room temperature, crystals of the penta-acetate had separated from the supernatant liquor. Water was added to dissolve the salt, and the crystals were collected, washed with water, and recrystallized from methanol in colourless needles (45 g.), m.p. 120°, $[\alpha]_D^{19°}$ +12·4° ($c = 5$ in tetrachloroethane). (Found: S, 8·0. Calc. for $C_{16}H_{22}O_{10}S$: S, 7·9%.) Glucothiose solution was prepared from this when required by deacetylation with sodium methoxide, and neutralization with acetic acid, followed by concentration under reduced pressure.

β-Mercaptopropionic acid. A solution of methyl acrylate (6 g.) in thioacetic acid (9 g.) was left at room temperature overnight and then distilled, to yield *methyl β-S-acetylmercaptopropionate* (9·8 g.) b.p. 68°/5 mm., $n_D^{17°}$ 1·4773. (Found: C, 44·55; H, 6·55; S, 20·3. $C_6H_{10}O_3S$ requires C, 44·4; H, 6·2; S, 19·8%.) A portion of this ester (9 g.) on saponification with cold 2N-NaOH overnight gave 3 g. of β-mercaptopropionic acid, b.p. 85°/5 mm., $n_D^{20°}$ 1·4918.

Ethyl mercaptosuccinate. Ethyl maleate (5 g.) was mixed with thioacetic acid (2·4 g.) and heated at 100° for 24 hr. Distillation of the product gave 5·4 g. of a *colourless oil*, b.p. 163°/14 mm. (Found: C, 48·3; H, 6·4. $C_{10}H_{16}O_5S$ requires C, 48·4; H, 6·45%.) Saponification of this ester gave mercaptosuccinic acid, m.p. and mixed m.p. 151°.

α-Mercaptopropionic acid. Ethyl α-bromopropionate (14 g.) was refluxed for 2·5 hr. in ethanol with potassium thioacetate (from 7·5 g. thioacetic acid). The solution was then poured into water and extracted with chloroform. The washed and dried extracts were evaporated and distilled, to give *ethyl α-S-acetylmercaptopropionate* (7·8 g.) b.p. 55°/3 mm., $n_D^{19°}$ 1·4635. (Found: C, 48·0; H, 7·2; S, 18·7. $C_7H_{12}O_3S$ requires C, 47·7; H, 6·9; S, 18·2%.) Saponification of a portion (7 g.) by shaking with cold 2N-NaOH overnight gave 2·3 g. of the acid, b.p. 102°/16 mm., $n_D^{16°}$ 1·4823.

α-Mercaptoglutaric acid. Ethyl α-bromoglutarate (9 g.) in ethanol (20 ml.) was treated with an ethanolic solution of potassium thioacetate prepared by neutralizing a solution of KOH (2 g.) in ethanol (20 ml.) with thioacetic acid. After standing overnight, water (50 ml.) was added, and the oil extracted with ether. Evaporation of the dried extract gave 9 g. of crude ester, a small portion of which was distilled at 178–179°/15 mm., and showed $n_D^{20°}$ 1·4727. The ester (8·5 g.) on saponification with cold aqueous ethanolic 2N-NaOH, gave *α-mercaptoglutaric acid* (2·8 g.) which after recrystallization from ether-benzene had m.p. 97°. (Found: C, 36·9; H, 4·9. $C_5H_8O_4S$ requires C, 36·6; H, 4·9%.) It gave a transient deep blue colour with aqueous ferric chloride and a positive nitroprusside reaction.

α-Mercaptoadipic acid. Similar treatment of ethyl α-bromoadipate (25 g.) gave *ethyl α-S-acetylmercaptoadipate*

(22 g.), b.p. 177°/12 mm., $n_D^{17°}$ 1·4680. (Found: equiv. by saponification, 91. $C_{12}H_{20}O_5S$ requires equiv., 92.) The ester, on saponification with aqueous ethanolic 2N-NaOH, gave *α-mercaptoadipic acid*, m.p. 112–113° after recrystallization from benzene. (Found: C, 40·5; H, 5·7; equiv. by titration with iodine, 180. $C_6H_{10}O_4S$ requires C, 40·4; H, 5·7; equiv., 178.) It gave a transient blue colour with ferric chloride. On standing in alkaline solution, exposed to the air, it readily gave *di(α-adipic acid) disulphide*; it had m.p. 180° after recrystallization from ether-ligroin, and gave no thiol reaction. (Found: C, 40·4; H, 5·3; equiv. by titration with NaOH, 88·5. $C_{12}H_{18}O_8S_2$ requires C, 40·7; H, 5·1%; equiv., 88·5.)

Allylglucoside. Glucose (300 g., anhydrous) was added to a 3% solution of HCl in dry allyl alcohol (1 l.) contained in a 2 l. 3-necked flask fitted with stirrer, inlet-tube and condenser. The mixture was heated to 70° and vigorously stirred for 2–3 hr., after which time only a small amount of glucose remained undissolved. Stirring was discontinued, and the internal temperature was then raised to 95° for a further 12 hr., HCl being passed into the boiling solution from time to time. After cooling, neutralization with barium carbonate, and filtration, the solution was evaporated first at ordinary pressure, and finally under reduced pressure, to a syrup; this was taken up in water and washed twice with ether. The syrup recovered by evaporation of the aqueous solution was then extracted five times with 1 l. portions of boiling acetone. Removal of the solvent from the combined extracts gave a non-reducing syrup (205 g.) which was used as such for the subsequent acetylation. Other preparations of the glucoside, allowed to stand for several weeks, gradually solidified, and recrystallization from acetone gave a mixture of α- and β-allylglucosides, m.p. 75–90°, $[\alpha]_D^{20°}$ +130° (c=1·1 in water). (Found: C, 48·9; H, 7·4. Calc. for $C_9H_{16}O_6$: C, 49·1; H, 7·3%.)

2:3:4:6-Tetra-acetyl allylglucoside. The above syrup (205 g.), with fused sodium acetate (100 g.) was dissolved in acetic anhydride (1 l.) and heated on the steam-bath for 1·5 hr. Most of the solvent was then removed, and the residual oil stirred with water to decompose any remaining anhydride. The product was then taken up in chloroform and washed with $NaHCO_3$ solution until neutral. Removal of the solvent from the dried extract gave the crude tetra-acetate (325 g.). In a subsequent preparation, the crude material was dissolved in ether and clarified by filtration through activated alumina. On evaporation of the filtrate, a solid residue was obtained, which was fractionally crystallized from light petroleum (b.p. 40–60°) to give *2:3:4:6-tetra-acetyl α-allylglucoside*, m.p. 52–53°, $[\alpha]_D^{20°}$ +115° (c=1·1 in methanol). (Found: C, 52·9; H, 6·3. $C_{17}H_{24}O_{10}$ requires C, 52·6; H, 6·2%.) From the mother liquors, the already known β form of m.p. 89° was obtained.

Tetra-acetyl dibromopropylglucoside. The crude tetra-acetate (325 g.), showing $[\alpha]_D^{20°}$ +96° (i.e. mainly the α-form) was dissolved in carbon tetrachloride (1 l.) and treated gradually with a solution of bromine (145 g.) in the same solvent, the temperature being kept below 25°. After washing with $NaHCO_3$ solution, the solvent was removed, to yield the syrupy dibromide (460 g.).

Tetra-acetyl β-(dibromopropyl)glucoside, m.p. 91°, was prepared from acetobromoglucose by the method of Fischer (1920).

BAL-INTRAV. (*First method.*) The crystalline β-dibromide (75 g.) in hot ethanol (200 ml.) was treated with ethanolic potassium thioacetate (from 25 g. thioacetic acid) and refluxed for 6 hr., after which water (1 l.) was added to dissolve the precipitated KBr. The oil, thrown out during this operation, was taken up in chloroform, washed, dried, and evaporated to a syrup (73 g.) which showed $[\alpha]_D^{21°}$ −18° (c=5 in methanol). This crude hexa-acetate, which could not be distilled without decomposition and which failed to crystallize, was dissolved in methanol (80 ml.) cooled to −5°, and well stirred during the addition of 1·13N-barium methoxide in methanol (300 ml.), the temperature being kept below 10°. More methanol (50 ml.) was then added to reduce the viscosity of the semi-solid reaction mixture, which was stirred for a further 15 min. and then filtered. The Ba salt was washed on the filter with methanol, well pressed down, and dried *in vacuo* over $CaCl_2$, and finally over P_2O_5. It showed $[\alpha]_D^{20°}$ −16° (c=10 in water). Other properties of this salt are recorded below.

BAL-INTRAV. (*Second method.*) This differed from the above only in the use of the syrupy dibromide (mainly the α-form) in place of the crystalline β-isomer. From 460 g. of this material, 400 g. of hexa-acetyl BAL-INTRAV were obtained, which was deacetylated as before to give the barium salt, $[\alpha]_D^{20°}$ +45° (c=2 in water). By the use of sodium methoxide for the deacetylation, the sodium salt of BAL-INTRAV was also prepared. Unlike the barium salt, it is soluble in methanol and is therefore precipitated from the reaction mixture by the addition of ethanol. Both salts are exceedingly hygroscopic when first collected on the filter, and in this state are very prone to undergo aerial oxidation. It is therefore important that washing should be carried out as expeditiously as possible. When thoroughly dry, the barium salt is quite stable in the air, but specimens of the sodium salt are usually still somewhat hygroscopic. Analytical results for some of the samples obtained have already been discussed (Table 5). On keeping in a stoppered bottle, the thiol content diminishes somewhat, probably owing to slight oxidation; e.g. a barium salt had an initial SH content of 9·8% which dropped to 8·2% after 14 months and a sodium salt contained 9·2% SH initially and 7·75% after 7 months.

Attempted hydrolysis of BAL-INTRAV. The barium salt (2 g., $[\alpha]_D^{20°}$ −16) was heated at 100° for 3 hr. with 2N-HCl (25 ml.). A viscous oil was gradually precipitated, and the observed rotation of the supernatant liquor changed to +1·30 (1 dm. tube); this solution took up a negligible amount of iodine; there was no evidence for the formation of BAL, but a control experiment, in which BAL (0·75 g.) glucose (1 g.) and 2N-HCl (25 ml.) were heated for 3 hr. at 100°, gave also a viscous oil, and the supernatant liquid contained only a trace of thiol. It is clear, therefore, that BAL itself is not stable under these conditions.

SUMMARY

1. Studies are described which resulted in the production of BAL-INTRAV (BAL glucoside), a thiol which is non-toxic when given by intravenous injection and which affords a high measure of protection against lewisite poisoning.

2. The toxicity of BAL, mercaptoacetate, mercaptosuccinate, mercaptoadipate, acetylmercaptosuccinate, dimercaptoadipate, glutathione, glucothiose and BAL-INTRAV (BAL glucoside) have

been studied when given by intravenous injection. In agreement with theory, increasing polarity of the molecule results in diminished toxicity. Mercaptosuccinate, acetylmercaptosuccinate, mercaptoadipate, dimercaptoadipate and glutathione cause no pathological symptoms in rabbits in dosage of up to 1 g./kg.

3. Many of these thiols when given by intravenous injection were able to prolong the life of rabbits given 3 LD_{50} of lewisite.

4. The most effective therapeutic action was obtained with BAL-INTRAV, which prevented death in 100% of the animals studied, even if not given until 6·5 hr. after administration of 3 LD_{50} of lewisite (which in untreated animals kills in 10 to 20 hr.).

5. Details are given of the preparation of mercaptosuccinic acid, dimercaptoadipic acid, glucothiose, BAL-INTRAV (BAL glucoside), and a number of other thiol acids.

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Changes Undergone by Phenylarsenious Acid and Phenylarsonic Acid in the Animal Body

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It is believed that many of the most important biological properties of the arsonic acids result from their partial reduction in the body to the corresponding arsenious acids. The evidence that this reduction does occur is, however, entirely indirect. Arsonic acids display a negligible parasiticidal effect *in vitro* and are effective *in vivo* only after a lapse of several hours following injection. The corresponding arsenious acids, on the other hand, are effective *in vitro*, and *in vivo* act immediately after injection (Voegtlin & Smith, 1920*a*; Yorke & Murgatroyd, 1930). Serum from animals injected with tryparsamide has been found to be trypanocidal *in vitro*, but the serum shows maximal activity only if it is withdrawn a long time after the injection of the arsonic acid. The arsenic content of the serum when

the trypanocidal activity is greatest must be a small fraction of the original value. In contrast, the trypanocidal activity of serum from animals injected with reduced tryparsamide dithioglycollate is greatest when the arsenic content is at its peak (Murgatroyd, Russell & Yorke, 1934). *In vitro*, Thuret (1938*a*) found that erythrocytes do not fix pentavalent, only trivalent, arsenicals. Following the injection of pentavalent arsenicals into the living animal, however, part of the blood arsenic is found attached to the erythrocytes (Thuret, 1938*b*).

If reduction of arsonic acids occurs in the body, it is reasonable to assume that oxidation of arsenious acids will also take place prior to their excretion in the urine, since arsonic acids are more rapidly excreted (Voegtlin & Thompson, 1923). From results obtained in a study of the fate of arsenic in the body following injection of rabbits with phenylarsenious acid, mapharsen (*m*-amino-*p*-hydroxyphenylarsenoxide), and the arsonic acid corresponding to each, it was postulated that the rates of excretion of the trivalent compounds were largely determined by the rates at which they were oxidized in the body to the arsonic acids (Chance, Crawford & Levvy, 1945). Since there is some evidence to suggest that the arsenobenzenes owe their therapeutic action to oxidation in the body to arsenious acids (Voegtlin & Smith, 1920*b*, *c*; Eagle, 1939; Rosenthal, 1932), information as to the fate of the latter is of interest also in connexion with the former class of compounds. In certain cases, at least, it is considered that the toxicity of organic arsenicals is partly due to the splitting off of inorganic arsenite or arsenate, but to what extent this occurs with compounds in common use in therapeutics it has been impossible to decide.

The lack of direct evidence regarding the changes undergone by organic arsenicals in the body results from the difficulty attending the separation in biological material of traces of the different compounds possibly formed. In a previous paper (Crawford, 1947), a method was described for the quantitative separation of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid in urine. After separation of the different fractions, the total arsenic in each was determined by a micro-titrimetric procedure (Levvy, 1943). The method has now been applied to an investigation of the metabolism of phenylarsenious acid and phenylarsonic acid in rabbits. These compounds may be regarded as the simplest prototypes of compounds in use in therapeutics, and were chosen for investigation since it was considered that the absence of substituents in the benzene ring might facilitate the study of the changes undergone by the arsenic-containing part of the molecule. It should be noted that, in spite of the unfavourable chemotherapeutic index, phenylarsenious acid is a very effective trypanocidal and treponemicidal agent (Eagle, Doak, Hogan & Steinman, 1940; Hawking, 1937). The initial assumption was made that any changes other than oxidation or reduction and decomposition to give arsenious or arsenic acids would be detected in the separation procedure. From the nature of the procedure, this assumption would appear to be sound.

METHODS

Solutions of phenylarsenious acid and phenylarsonic acid as their sodium salts were prepared and tested as described in an earlier paper (Chance, Crawford & Levvy, 1945). Male rabbits only were used. After administration of the arsenicals by intravenous injection, the rabbits were placed in metabolism cages and the urine collected in flasks, containing 10 ml. N-H_2SO_4 and 2 ml. chloroform, which were changed every 24 hr. Control experiments showed that, under the conditions of the urine collection, partial oxidation of phenylarsenious acid sometimes occurred even in the presence of chloroform unless acid was also present. At the end of each period of urine collection, the animal was catheterized and the urine thus obtained added to that already collected. Urine was stored in the refrigerator until it could be analyzed. Cages were well washed between successive periods of urine collection.

A measured volume of urine (40–200 ml.) was dialyzed against 200–310 ml. distilled water containing a few drops of chloroform for 24 hr. in the refrigerator. Experiments in which the urine was submitted to exhaustive dialysis showed all the As excreted to be dialyzable. Samples of dialysate were taken for fractionation according to the method already described (Crawford, 1947). In experiments with phenylarsenious acid, and in the later stages of the phenylarsonic acid experiments, the amounts of As excreted were so small that three days' urine was pooled for dialysis, and it was necessary to use more than 50 ml. of dialysate for each of the two parts of the fractionation procedure. Evaporation *in vacuo* of the dialysate could not be done since control experiments showed that such a procedure led to valency changes in the As. It was found, however, that the analytical method could be applied to volumes of fluid larger than 50 ml. provided corresponding increases were made in the amounts of the reagents. Increasing the amount of dithiol reagent proved unnecessary. In the phenylarsonic acid experiments the As contents of the urine dialysates were usually so high that the whole fractionation could be carried out in triplicate. As was determined in the sac contents at the end of the dialysis period so that the total in the urine could be calculated. To check the amounts found in various fractions in the separation, direct analysis of the original urine samples for total As was also done whenever material permitted.

RESULTS

The results obtained in the fractionation of urine from male rabbits injected with phenylarsonic acid (two doses) and phenylarsenious acid (one dose) are summarized in Table 1. The amounts of drug injected are expressed in mg. As/kg. body weight.

Table 1. *Nature of the urinary As after intravenous injection of phenylarsenious acid and phenylarsonic acid into rabbits*

Exp. no.	Wt. of rabbit (kg.)	Urine collection period (hr.)	Dose of arsenical (mg. As/kg.)	Fraction of dose excreted in urine (%)	Percentage urinary As as: Phenylarsenious acid	Phenylarsonic acid	Arsenious acid	Arsenic acid	Unidentified form
				Phenylarsonic acid					
1	2·36	0–24	1·11	25	Tr.*	92	Nil	Nil	Nil
2	2·34	0–24	1·11	70	Tr.	93	Nil	Tr.	Nil
3	2·50	0–24	1·11	71	Tr.	93	Nil	Nil	Nil
4	2·30	0–24	5·56	70	9	91	Nil	Nil	Nil
5	1·92	0–24	5·56	79	8	92	Nil	Nil	Nil
6	2·48	0–24	5·56	80†	8	92	—	—	—
7	1·60	0–24	5·56	54†	Tr.	96	—	—	—
		24–72		2†	23	77	—	—	—
8	1·85	0–24	5·56	60†	8	92	—	—	—
		24–96		1	Tr.‡	73	Nil.	Nil	Tr.‡
9	1·87	24–96	5·56	1	Nil	100	Nil	Nil	Nil
				Phenylarsenious acid					
10	2·85	0–72	0·45	16	Nil	48	Nil	Nil	52
11	2·15	0–72	0·45	15	Nil	45	Nil	Nil	55
12	2·05	0–72	0·45	6	Nil	56	Nil	Nil	44
13	2·27	0–72	0·45	9	Nil	52	Nil	Nil	48

* Tr. = trace.
† Dialysate extracted with the dithiol reagent only (see text).
‡ The small amount of As not found in the phenylarsonic acid fraction was equally divided between the phenylarsenious acid fraction and the aqueous residue from the fractionation.

Amounts of As in each fraction are shown as percentages of the total As in the urine. The concentration of As in the first 24 hr. specimen of urine in the phenylarsonic acid experiments was sufficiently high to permit of analyses in triplicate and the figures shown in the table are the mean values. In all the other experiments a single fractionation only was possible. When the amount of As in the final determination, while outside the 'blank' range, was so small that the analytical figure had no quantitative significance ($>4\mu$g.), the result for the appropriate fraction is shown in the table as 'trace'.

No As was excreted in the urine as arsenious acid or arsenic acid after injection of phenylarsenious acid or phenylarsonic acid (Table 1). In only one experiment was any As found in the 'inorganic' fractions and, in that, the amount was so small as to be of little significance. After the injection of phenylarsonic acid, most of the As appeared in the urine in the form in which it was administered. Almost always, however, a small fraction of the As appeared in the phenylarsenious acid fraction. After allowing for possible entrainment of up to 5% of the phenylarsonic acid during extraction with the dithiol reagent, the results still suggest that phenylarsenious acid was present in appreciable amounts in many of the samples of urine. Since all As excreted after injecting phenylarsonic acid was found in early experiments to be in the phenylarsenious acid and phenylarsonic acid fractions, it was found possible to employ the 'dithiol reagent' (a solution of ethane-1:2-dithiol in carbon tetrachloride, Crawford & Levvy, 1946) alone for fractionating the excretion products of this arsenical, and this was actually done in three of the later experiments. It was not possible, however, to simplify the fractionation procedure in this way after injecting phenylarsenious acid, since half the As present was in a form which did not react as arsenious acid, arsenic acid, phenylarsenious acid or phenylarsonic acid. All that one can say as to the nature of the unidentified As is that it was not extracted from acidified urine dialysate with ether after reduction or with the dithiol reagent before or after reduction. Attempts to convert it into arsenious acid, arsenic acid, phenylarsenious acid or phenylarsonic acid by incubating the urine dialysate at 38° at pH 10 for 3 hr. were unsuccessful. Qualitative studies were impossible with the minute amounts of material available. The largest amount of the unidentified arsenical present in three days' urine corresponded to only 106 μg. As.

DISCUSSION

After the injection of rabbits with phenylarsenious acid, no detectable amounts of arsenious acid or arsenic acid were found in the urine; approximately half was probably present in a form which reacted as phenylarsonic acid and the remainder in an unidentified form. These results confirm to some extent the suggestion made in an earlier paper (Chance, Crawford & Levvy, 1945) that the rate of oxidation

of an arsenious acid to the corresponding arsonic acid determines the rate of its excretion in the urine, the latter in turn determining the toxicity of the arsenical (Voegtlin & Thompson, 1923). The presence and nature of the unidentified arsenical in urine from rabbits injected with phenylarsenious acid emphasizes the risk involved in assuming that any As not extracted by an agent reacting with a trivalent arsenical (Crawford & Storey, 1944; Crawford & Levvy, 1946; Graham, Crawford & Marrian, 1946) is the corresponding pentavalent compound without further examination.

Most of the As in the urine after injection of phenylarsonic acid was in a form which reacted as phenylarsonic acid. No arsenious acid or arsenic acid was detected but a small fraction (not more than 10%) of the As appeared to be present as phenylarsenious acid. It is interesting to note that the amount of phenylarsenious acid excreted was, however, sufficient to account for the toxicity of phenylarsonic acid on the theory that the latter must be reduced in the body before producing its lethal effects. In terms of elemental As the lethal dose of phenylarsenious acid is one-seventeenth that of phenylarsonic acid (phenylarsonic acid, LD_{50} 5·94 mg. As/kg. (Hogan & Eagle, 1944); phenylarsenious acid, LD_{50} 0·35 mg. As/kg. (Eagle *et al.* 1940). If the toxic effects of phenylarsonic acid are entirely secondary to the formation of phenylarsenious acid, it is difficult to explain the absence of the unknown arsenical, formed from the latter, from the urine of animals injected with the former. The absence of any detectable amounts of phenylarsenious acid in the urine after injection of this substance is not necessarily in contradiction with its presence after injecting the arsonic acid. If the ratio of phenylarsenious acid to phenylarsonic acid in the urine were the same in both cases, the trivalent fraction after injecting the arsenious acid would not be measurable since the total amount of As excreted was so small.

In the earlier paper (Chance, Crawford & Levvy, 1945), it was shown that As injected into rabbits as phenylarsonic acid was cleared almost exclusively in the urine, while in the case of phenylarsenious acid a small fraction of the As excreted was found in the faeces. Figures for the total As in the urine were in general similar to those given in Table 1.

In so far as they suggest that a trivalent arsenious acid and the corresponding pentavalent compound are interconvertible in the body, the results described above agree with those obtained by Storey (unpublished work), who applied the 'xanthate reagent' (a solution of sodium ethyl xanthate in carbon tetrachloride, Crawford & Storey, 1944) to a study of the valency of the As derivatives in the urine of rabbits injected with arsenious acid or arsenic acid. After injection of either compound, the bulk of the excreted As was not extracted by the 'xanthate reagent', but a small fraction was present in the trivalent state in both cases.

SUMMARY

1. The nature of the arsenicals excreted in the urine of rabbits injected with phenylarsenious acid and phenylarsonic acid has been studied.

2. No detectable amounts of arsenious acid or arsenic acid were present after the injection of phenylarsenious acid or phenylarsonic acid.

3. After injection of phenylarsonic acid, most of the arsenic in the urine appeared to be in the form in which it was administered. A small fraction (not more than 10%) was, however, in a form which reacted as phenylarsenious acid.

4. Of the arsenic excreted in the urine after injecting phenylarsenious acid, 50% appeared to be present as phenylarsonic acid and the remainder in the unidentified form.

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Studies on the Reaction between Sodium Hypochlorite and Proteins

1. PHYSICO-CHEMICAL STUDY OF THE COURSE OF THE REACTION

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In connexion with their application in surgery, a comprehensive investigation of the action of solutions of sodium hypochlorite on proteins is being undertaken. The reactions have been studied first from physico-chemical standpoints, using egg albumin.

Earlier work on this subject is reviewed by Engfeldt (1922). More recently, Price (1931) has dealt with the nature of the reaction between sodium hypochlorite and gelatin. Wright (1926, 1936) emphasized the relative parts played by chlorination and by oxidation. Lieben & Bauminger (1933) also found, with casein, that the protein was both oxidized and chlorinated in alkaline solution by sodium hypochlorite. Commercial interest has led to work on the reaction between sodium hypochlorite and proteins (e.g. Cross, Bevan & Briggs, 1908; Briggs, 1918; Trotman, Trotman & Brown, 1928).

Reactions between sodium hypobromite and proteins, peptones, amino-acids and 'model-compounds' have received extensive attention in Germany by Goldschmidt *et al.* (e.g. Goldschmidt & Strauss, 1929) and by Brigl, Held & Hartung (1928).

This earlier work indicates that: (1) amino-acids and the amino-acid residues in proteins are attacked by sodium hypochlorite at speeds which differ greatly from one such acid or residue to another; (2) groupings other than the 'peptide linkage' in proteins are attacked; (3) both chlorination and oxidation occur; (4) groupings of different types comprising 'active chlorine' are formed. These groups show widely divergent degrees of stability; (5) the nature of the reaction does not vary greatly from one protein to another, and changes in concentrations and temperature bring about only changes of degree; (6) in distinctly acid solution, chlorination predominates over oxidation, and the nature of the reaction varies little over the range pH 1–6. In alkaline solution oxidation is more marked, but the reaction is profoundly influenced by the concentration of hydroxyl ions.

Preliminary unpublished work in the present investigation showed that albumin reacted rapidly with sodium hypochlorite with evolution of heat, and that as the reaction proceeded the pH of the reaction mixture rose sharply at first before exhibiting the fall reported by other authors (e.g. Norman, 1936).

It was found, also, that casein and solid heat denatured egg albumin gave protein- and peptone-like bodies of comparatively high solubility, together with products of much lower molecular weight. In all fractions of the reaction mixture containing 'Kjeldahl nitrogen', combined electropositive chlorine was detected. The protein-fraction resembled somewhat the chloro-protein described by Salkowski (1923), and the bromo-protein precipitated by acetic acid from alkaline solution (Vandervelde, 1924). The absence of chlorate from the final reaction mixture was established, after precipitation of peptone, etc., by the ferrous sulphate—permanganate method.

The work now described falls into three parts: (A) A curve was constructed to show titres of electropositive chlorine, determined iodometrically, at different stages during the reaction of sodium hypochlorite with egg albumin in alkaline solution. Corresponding figures were obtained for the amount of nitrogenous (Kjeldahl) material that could be precipitated by phosphotungstic acid, thus giving a curve representing the progressive degradation of protein as a function of time. (B) Curves for temperature against time were constructed for the reaction. (C) Curves for pH value against time were constructed.

EXPERIMENTAL

A. *Degradation of protein*

Methods. The hypochlorite solution ('Milton') of which 101 ml. were taken, contained 1·12 g. NaOCl and 18·65 g. NaCl/100 ml. It was stirred mechanically in a thermostat at 36° and two samples of 0·5 ml. each were withdrawn for analysis, using KI solution and acetic acid, followed by titration with 0·02 N-$Na_2S_2O_3$. The reaction with protein was started at zero time by running in 25 ml. of solution containing 1 g. of granular egg albumin (moisture + ash = 14·5%) in 0·5% NaCl solution. Samples of convenient volume (1–2 ml.) were withdrawn successively over the first hour and analyzed as above. A final sample was titrated after 19 hr.

Degradation was followed by treating reaction-mixtures as above after different times to recover material of the

protein-to-peptone class: At the predetermined time, 20 ml. of KH_2PO_4 buffer (0·154 M, pH 4·6) were added and SO_2 was bubbled through, avoiding excess, until the reaction of starch-KI paper was negative. Phosphotungstic acid (2% in 1·5 N-H_2SO_4, 25 ml.) was stirred in and after 15 min. the precipitate was separated by decanting and centrifuging. It was washed once with 20 ml. of a liquid of the composition: 130 ml. 11·3% NaCl solution + 19 ml. phosphotungstic precipitant + 20 ml. phosphate buffer (as above) and again centrifuged. Original supernatant liquids and washings showed no biuret reaction. The solid was taken up with 14 ml. of 2 N-Na_2CO_3 and made up with water to 25 ml., from which duplicate samples of 0·64 ml. were taken (microburette) for Kjeldahl analysis in which the ashing and the indicator were as described by Miller & Houghton (1945); NH_3 was distilled into 0·143 N-HCl from 40% NaOH containing $Na_2S_2O_3$. Excess acid was titrated with 0·143 N-NaOH. Results for total N of the precipitate were recorded as percentages of the value found in a control experiment in which NaCl (16·5%) was used in place of the hypochlorite. Duplicate results were reproducible within 1·2% (overall) of the mean. Duplicate determinations of N differed by less than 0·3% from the mean. The control experiment showed that the recovery of N was 88% of the calculated value.

Results. These (Fig. 1) show a close correspondence between the rapid reduction of hypochlorite and the degradation of protein. It is calculated that after

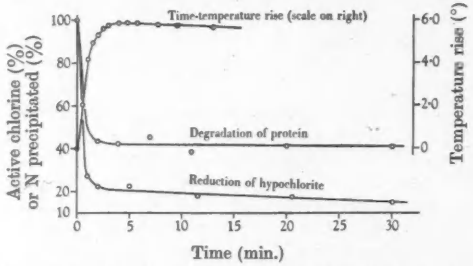

Fig. 1. Exothermic, oxidative degradation of albumin by NaOCl at 36°. 1·12 g. NaOCl + 1·0 g. albumin (84·5%) in 125 ml.

10 min., when an almost steady state has been reached, for each mol. of NaOCl reduced, 33·7 g. of albumin are rendered incapable of coprecipitation with phosphotungstic acid, while the remainder of the original protein (22·5 g.) was found by qualitative examination to be changed to polypeptide precipitable by phosphotungstic acid but not precipitated by trichloroacetic acid or saturation with $(NH_4)_2SO_4$. The reduction of hypochlorite is closely coupled also with the exothermic effect as seen from the temperature-time curve (Fig. 1).

B. *The exothermic reaction*

Methods. Reactions with albumin and NaOCl (as above) were carried out in a silvered vacuum vessel with mechanical stirring. Temperatures were measured with a thermometer reading to 0·1°. Before an experiment, water (250 ml.) at 34° was stirred in the vessel until thermal equilibrium was reached. The water was removed by suction and the hypochlorite solution, with or without buffer, in a total volume of 200 ml., and at the equilibrium temperature, was introduced. A cooling curve was constructed for the course of 5 min. The albumin solution (25 or 50 ml.), adjusted to the zero-time temperature, was added, and temperatures were recorded at suitable intervals over the course of about 12 min. Trials with water alone showed that the error due to incorrect matching of the temperature of the albumin solution was less than 0·03°, and that fluctuation was smoothed out within 15 sec.

With the total volume and the quantity of the second reagent fixed, experiments were carried out using different quantities of protein and of hypochlorite. A hypochlorite solution containing 1·3% of NaCl was then used to detect any effect due to the high salt concentration generally employed. No such effect was observed.

For reactions in the presence of buffer, phosphate (0·068 M in the reaction mixture) was used in the pH range 6·8–10·0. In view of the statement (Mauger & Soper, 1946) that phosphate takes part in *N*-chlorination, temperature curves were repeated with more dilute phosphate (0·0171 M) and with 0·4 M-carbonate—bicarbonate buffers of approximately the same pH values.

Comparable experiments were carried out at pH 9·0 and 5·5 with *N*-methylacetamide solution (2·78%) of the same N content as the albumin solution used.

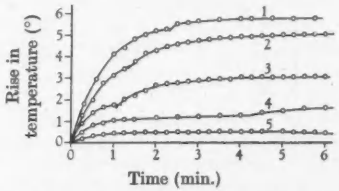

Fig. 2. Exothermic reaction of albumin with NaOCl. 2·24 g. NaOCl + x g. albumin in 250 ml., initially at 32°. x = (1), 2·0; (2), 1·4; (3), 0·8; (4), 0·4; (5), 0·2.

Table 1. *The effect of varying hypochlorite concentrations on the rise of temperature in the reaction between egg albumin and sodium hypochlorite*

(Reaction system: 50 ml. 4% albumin in 0·5% NaCl solution + x ml. NaOCl solution (1·12 g. NaOCl + 18·5 g. NaCl/100 ml.) + (200 − x) ml. NaCl solution (18·5 g./100 ml.). Initial temperature: 34°.)

x	200*	100	70	50	30	10
Maximum rise (°)	5·63	2·54	1·87	1·32	0·63	0·06
Initial rate of heating (°/min.)	10·0	6·3	6·0	5·4	3·4	0·9

* Secondary rise apparent: cf. Fig. 2.

Results. In the absence of buffer, the reaction appears to proceed in two distinct stages when relative excess of oxidizing agent is used (Fig. 2). With excess of protein, however, smooth curves with the characteristics given in Table 1 were

obtained. Time-pH curves for such reactions are discussed in the next section.

The rate of evolution of heat was directly proportional to the concentration of (*a*) protein, and (*b*) hypochlorite, when one of the reagents was present in excess (Fig. 6). The linear relationship with respect to protein was observed when the weight of NaOCl was not less than 2·8/unit wt. of protein taken. The linear relationship with respect to hypochlorite was maintained, while the same weight ratio did not exceed 0·3:1·0.

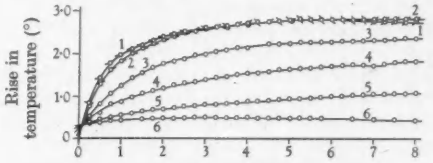

Fig. 3. Exothermic reaction of albumin with NaOCl at different pH. NaOCl (1·12 g.) + 1·0 g. albumin in 250 ml. solution. 0·0616 M-phosphate; 32°; initial pH: (1), 9·6; (2), 9·0; (3), 8·5; (4), 7·7; (5), 7·3; (6), 6·9.

With the buffered system, the exothermic reaction varies greatly with pH (Fig. 3); and it also appears that two distinct reactions occur, since in all cases where the initial pH is less than 8·9 an instantaneous rise in temperature is found, followed by the slower and more strongly exothermic reaction. The rapid heating effect appears to be due to a reaction catalyzed by hydrogen ions (Table 2) since it increases almost linearly with decreasing pH value.

Table 2. *The catalysis of the reaction by hydrogen ions*

Initial pH	9·60	8·95	8·50	7·60	7·25	6·85
Instantaneous rise in temp. (°)	0·00	0·00	0·10	0·24	0·29	0·33

Results of both experiments with *N*-methylacetamide (pH 9·0 and 5·5) are different from those with protein. In alkaline medium, a rise of not more than 0·05° was found, with no further change after 1 min. In acid solution the rise during the first minute, after which no further change was observed, was 0·18°.

C. *Changes in* pH *value during the reaction*

Methods. Hypochlorite and 4% albumin solutions were the same as above. The former (100 ml.) was stirred mechanically in a wide-necked bottle in a thermostat at 36°. A glass electrode, an immersion-type calomel half-cell and a thermometer were dipped into the liquid. pH values were determined (Cambridge pH meter) for 5 min.; the reaction was then started by introducing 25 ml. of the protein solution, and pH values were measured at intervals for 10–15 min. A glass electrode adapted for use in high ranges of pH values (Cambridge 'Alki') was used, after early trials with the ordinary type of glass electrode had given consistent but erroneous results with both reaction mixtures and alkaline buffer solutions. Tests with buffer solutions showed that the 'Alki' electrode gave a true reading within a few seconds and that the instantaneous value differed by only 0·4 pH unit from the equilibrium figure. Adequate earthing of metal apparatus was found to be essential. Erratic readings found initially were due to a calomel element whose appearance and behaviour when used in conjunction with an ordinary glass electrode, did not reveal any fault. Immediately before each experiment the electrode system was standardized against borate buffer of pH 9·10 at 36°; checks after experiments showed drifts never exceeding 0·03 pH. A series of curves were constructed for reaction mixtures of different ratios and concentrations of reagents. A second series was constructed with phosphate buffers in the mixture.

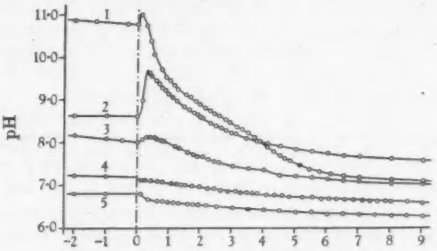

Fig. 4. Variation of pH with time for reactions of albumin with NaOCl in buffered solutions. 1·12 g. NaOCl + 1·0 g. albumin in 250 ml. at 36°. (1), no buffer; (2)–(5), 0·0616 M-phosphate.

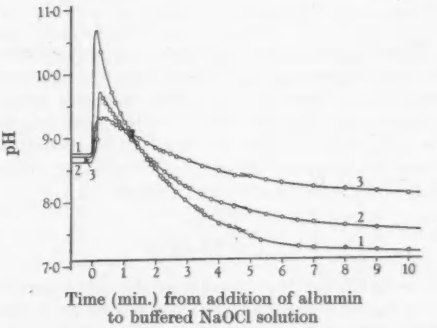

Fig. 5. Variation of pH with time for reactions of albumin with NaOCl in buffered solutions. 1·12 g. NaOCl + 1·0 g. albumin in 250 ml. at 36°. (1), no buffer (pH adjusted with 2N-H_2SO_4); (2), 0·0616 M-phosphate; (3), 0·246 M-phosphate.

Results. (Figs. 4 and 5.) When the initial pH of the reaction mixture is 8·5–9·0, a considerable increase in alkalinity is found, followed by an

approximately smooth and relatively large fall. The magnitudes of rise and fall decrease as the initial pH is made to approach 7·5. On the acid side of this point the initial effect is inverted. Results for buffered and for adjusted reaction mixtures are shown in Fig. 5. Five experiments with the reaction mixture x ml. hypochlorite solution + $(100-x)$ ml. water + 25 ml. albumin solution gave curves whose slopes, immediately following the initial rise in pH, are directly proportional to x (Fig. 6).

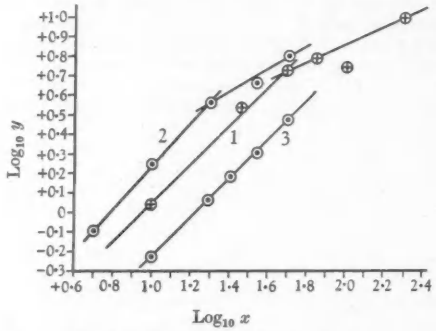

Fig. 6. Reaction between NaOCl and albumin (varied amounts) in 250 ml. at 36°. Rates of exothermic reaction and of falls in pH.

(1) x = ml. NaOCl (0·16 M) to 2·0 g. albumin (84·5%). y = initial rate of rise in temperature (° C/min.).

(2) x = ml. 4% albumin solution to 2·24 g. NaOCl. y = initial rate as in (1).

(3) x = ml. NaOCl as in (1). y = initial rate of fall in pH value from maximum attained.

To estimate the amount of added base represented by the increase in pH value shown in Fig. 5, curve 2, suitably diluted buffered hypochlorite was titrated electrometrically with 0·1 N-NaOH. The increase in pH value in the reaction with protein was found to be equivalent to the addition of 17·5 ml. 0·1 N-NaOH to the reaction mixture.

DISCUSSION

The results of the thermal study emphasize the complexity of the reaction. Three reactions appear to have been distinguished; two in alkaline solution, and a third which takes place only in acid or weakly alkaline conditions. The second alkaline reaction is found only when the weight ratio of NaOCl to albumin exceeds 1·3 : 1, i.e. with relative excess of hypochlorite, and when the pH value is initially greater than 9·6. That such a rapid reaction should set in after at least a minute, and only with sufficient hypochlorite, suggests that a product of the first alkaline reaction is involved. From measurements of slopes, both alkaline reactions are found to proceed at rates which are approximately proportional to the initial concentration of protein. The variation of the rate with pH (Fig. 7) supports the

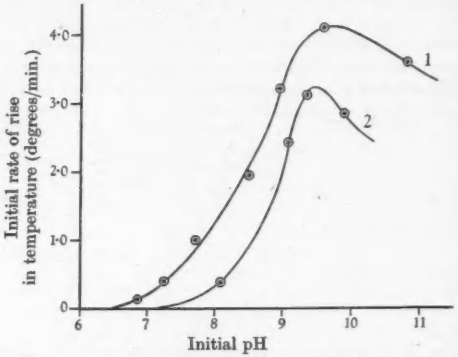

Fig. 7. Rate of exothermic reaction as function of initial pH. (1), 0·068 M-phosphate. (2), 0·0171 M-phosphate or 0·4 M-carbonate-bicarbonate.

conclusion of previous workers (Price, 1931; Wright, 1936; Norman, 1936). The work of Mauger & Soper (1946) is also confirmed qualitatively within the limits of the present data. The third reaction mentioned above is very rapid and its effect decreases as the pH is increased (Table 2) to an extinction point at pH 9·0. All three reactions are considered to be oxidations in view of the close connexion with the reduction of hypochlorite and their strongly exothermic nature.

The evolved heat as measured in part (B) is made up of various components. In chlorination, hydroxyl ions are produced. In the oxidation of protein, new carboxyl groups are formed and ammonia and other basic substances may appear. Thus, in addition to the heat evolved in the oxidation proper there is the heat ascribable to ionic reactions consisting of mutual neutralization of products and to the reaction of excess acid with buffer. Such secondary effects should be corrected for, if the oxidation is to be followed by thermal measurements.

Since the cleavage of protein in the conditions used is oxidative, while hydrolytic action, as shown by Engfeldt (1922), is negligibly small, it is assumed that basic groups are produced in very small amounts only. Thus, as a first approximation, correction of the total heat evolved is concerned only with the heat due to reaction of the new acidic groups with the buffer:

$$—COOH + OH^- \longrightarrow —COO^- + H_2O,$$

and with neutralization of the base formed in chlorination:

$$OH^- + H^+ \longrightarrow H_2O.$$

The heat of formation of water is 13,700 cal./equiv. Since, as found above, the amount of alkali produced by chlorination is equivalent to approximately 17·5 ml. 0·1N in the reaction of 1·0 g. protein with 1·12 g. sodium hypochlorite, the neutralization of this amount of alkali contributes 24 cal., equivalent in the experimental conditions to a rise in temperature of not more than 0·1°, i.e. approximately 3% of the total rise in temperature recorded. If it is assumed that one carboxyl group is produced for each peptide link broken, i.e. for each 2·9 mol. of sodium hypochlorite reduced (see below), the maximum error is not greater than 0·3°, i.e. 10%. This is considered to be an outside figure since the value of 2·9 taken above includes hypochlorite reduced in side reactions. The total error in the thermal measurements is therefore not greater than 15%, and will be little in excess of 10% in reactions at pH values nearer 7·0 since chlorination as shown by the initial rise in pH is now diminished.

Results with *N*-methylacetamide indicate that the behaviour of the —CONH— grouping in this compound is not in any way comparable with its behaviour in proteins. Thus, the same linkage in protein would appear not to suffer oxidation (Goldschmidt & Steigerwald, 1925). Peptide links, however, differ from the —CONH— grouping in *N*-methylacetamide since in the structure —CONH.CHR.CONH— the CHR— and —NH— groups are under the influence of two electronegative groups.

There are important groups of minor abundance in the albumin molecule which may be oxidized by sodium hypochlorite. The sulphur linkages in cystine (and cysteine) and perhaps even in methionine, the cyclic nitrogenous systems in histidine, tryptophan and proline, free amino-groups in, e.g. lysine, the guanidino grouping in arginine, and the aromatic group of tyrosine are all liable to rapid attack. The aromatic ring of phenylalanine, and hydroxyl groups as in serine probably react but slowly with sodium hypochlorite, and carboxyl groups as in glutamic acid are probably fairly inert towards this reagent.

The initial increase in pH value in the reactions can only be due to decarboxylation of free acidic groups or to *N*-chlorination:

$$\rangle NH + NaOCl \rightarrow \rangle NCl + NaOH.$$

In view of the relatively inert nature of aliphatic acids towards sodium hypochlorite (Engfeldt, 1922), the first supposition is excluded. Further, the maximum pH value is reached before any significant reduction of hypochlorite has occurred. Difficulty in preventing the early rise in alkalinity with buffer shows that a strong base is liberated. The chlorination is therefore put forward as an explanation of the effect observed.

Complete reaction with excess of chlorine-acceptor would, with the amount of hypochlorite taken, lead to the formation of sodium hydroxide equivalent to approximately 150 ml. 0·1N. The electrometric titration shows that alkali appears in an amount which is 12% of this value, and it is inferred that active chlorine represented by at least 12% of the original quantity taken is present within a short time in combined form. Subsequent oxidation would depend on progressive hydrolysis of the 'bound active chlorine', leading to production of acid:

$$\rangle NCl + H_2O \rightarrow \rangle NH + HOCl; \quad HOCl \rightarrow HCl + O.$$

The overall effect is therefore:

$$NaOCl \rightarrow NaCl + O.$$

Since the ultimate pH value is lower than the initial figure either organic acid is produced by the oxidation or basic groups are destroyed, or both. It is well known that amines and ammonia are oxidized by hypochlorites in alkaline solution, whereas aliphatic acids are not in general attacked (Engfeldt, 1922). If both acids and nitrogenous bases are produced by oxidative fission of the protein the bases will be destroyed by subsequent action of the hypochlorite and the acids remaining will effect a lowering in the pH of the reaction mixture. Close correspondence between pH- and temperature-time curves suggests that the exothermic oxidation results in a lowering of the pH of the reaction mixture.

It is noted (Cohn & Edsall, 1943) that a well-defined break in the titration curve of egg albumin is found at pH 8·5 and this is attributed to free amino-groups. Such groups are probably oxidized readily by hypochlorite and much further work is necessary to allow of a full explanation of the secondary breaks in the present experimental curves.

From quantitative data on the amino-acids obtained by hydrolysis of egg albumin (Block, 1945) the average mol. wt. $\overline{m}$ of an amino-acid is 116·4. In the protein molecule each acid is represented by its residue. If terminal groups are neglected the average residue weight is that of the acid less that of water, i.e. 98·4. If bonds such as —S.S— are disregarded and albumin is considered as a peptide chain of molecular weight 34,000, and if it is assumed that the protein is degraded either to a peptone of molecular weight 2000 or to substances of average original molecular weight 98, it is calculated from the results of (A) that for each peptide link broken, 2·9 molecules of sodium hypochlorite are reduced. The less accurate average value for this quantity given by the limits of concentrations for 'linear' exothermic reaction (break-points in curves 1 and 2, Fig. 6) is 2·5, if 98 is taken as the average residue weight.

SUMMARY

1. Reaction between egg albumin and sodium hypochlorite results in degradation of protein and reduction of hypochlorite accompanied by evolution of heat, all of which follow a similar course. There is no significant change after ten minutes.

2. From thermal data it is concluded that three oxidations occur. Oxidation is most rapid at pH 9·8.

3. The pH falls as the reaction proceeds: in alkaline solution there is first a rise, and this rise increases with greater initial alkalinity.

4. Approximately 2·9 molecules of NaOCl are reduced for each amino-acid residue attacked.

5. *N*-Chloro-groups form rapidly and hydrolyze as the oxidation proceeds.

6. *N*-Methylacetamide is not oxidized significantly by sodium hypochlorite under the conditions used.

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Antidotal Activity of British Anti-Lewisite against Compounds of Antimony, Gold and Mercury

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Ever since the discovery of 2:3-dimercaptopropanol (British Anti-Lewisite, or BAL) as an arsenical detoxicant (Stocken & Thompson, 1940, 1941; Peters, Stocken & Thompson, 1945), its application as a possible antidote for compounds of other metalloids and metals had been envisaged in this laboratory, and a few experiments were tried at an early date. For various reasons it was not possible to report this work till 1945 when Whittaker (1945*a*) submitted an account of various aspects of the problem to the Ministry of Supply.

Meanwhile, reports had appeared from America on the effects of BAL on the toxic actions of Bi, Hg, Cd, V and Se (Barron & Kalnitsky, 1944), Cd (Sulzberger & Baer, 1944) and Sb (Calvery, Braun & Lusky, 1944), and in this country on effects obtained with Zn (Bruner, 1945).

From the earlier work with arsenic, theoretical considerations alone rendered it likely that antidotal effects should be obtained with antimony and also with divalent toxic metals such as copper or mercury. As evidence had been obtained, however, showing that a considerable proportion of cases of post-arsphenamine dermatitis responded clinically to a course of injections of BAL (Carleton, Peters, Stocken, Thompson, Williams, Storey, Levvy & Chance, 1946) it was decided to study first those metals whose compounds are used therapeutically. The work described here therefore deals only with antimony, gold and mercury, and chiefly with the organic compounds of these metals in use in medicine at the present time.

Extension of this work to the treatment of the dermatitis that develops occasionally in patients receiving therapy with compounds of gold has already begun.

As in the earlier work with arsenic, the initial experiments were designed to test the effect of BAL

in counteracting the inhibition of the brain pyruvate oxidase system caused by the presence of compounds of these metals. All the metal compounds described below were found to inhibit this system much less than either lewisite or mapharside; consequently, the 'brain dispersion' preparation, used by Whittaker (1945*b*), in experiments with arsphenamine and neoarsphenamine, has been used for most of the experiments. In addition to enzyme work, rat survival experiments are now being carried out with tartar emetic.

EXPERIMENTAL

Two types of pyruvate oxidase preparation have been used: pigeon brain *brei* (Passmore, Peters & Sinclair, 1933) and pigeon brain dispersion (Banga, Ochoa & Peters, 1939). Activity was determined by the Warburg technique in the presence of sodium pyruvate at pH 7·3 and 38°, as described by Stocken, Thompson & Whittaker (1947).

Both protection and reactivation experiments have been carried out; in the former, done with the *brei* preparation, the BAL, freshly dissolved in Ringer-phosphate, was added to the brain sample dispersed in the Ringer-pyruvate mixture, the metal compound being added 2–3 min. later; when the dispersion was used the enzyme was added to the Ringer-phosphate already containing thiol, metal compound and substrate. In the reactivation experiments the BAL was placed in the side-bulbs, and the metal compound added to the unprotected brain in the main compartment of the flask. The manometers were then attached to the thermostat, and 15 min. allowed for temperature equilibration at 38°, after which the side-bulb contents were tipped into the main compartment at measured times, and the effect of this addition on the inhibition was noted over a further measured period of time.

RESULTS

Antimony. Three therapeutic antimonials were selected: potassium antimonyl tartrate (tartar emetic), 'stibophen' (a complex compound of trivalent antimony and sodium catechol disulphonate) and 'stibacetin' (a compound containing pentavalent antimony, sodium *p*-acetylaminophenylstibonate). These three antimonials differ in their toxicity to the pyruvate oxidase dispersions, pentavalent antimony, like pentavalent arsenic, being very much less toxic than trivalent compounds. Thus, in Table 1, stibacetin is seen to have less than one-tenth of the toxicity of stibophen, and less than a fortieth of the toxicity of tartar emetic. Table 1 also shows that BAL is able to protect the enzyme system from inhibition by tartar emetic or stibophen when present in a ratio of 6 mol. of BAL : 1 of antimonial. Molecular weights for stibophen and stibacetin were calculated from the formulae given by Fischl & Schlossberger (1934). An attempt was made to detoxicate stibacetin, but owing to the relatively high concentrations of this compound required to inhibit the enzyme system, the concentrations of BAL needed to detoxicate were so great as to produce significant inhibitions themselves.

Table 2 shows some reactivation experiments with tartar emetic. In these experiments readings were taken at the end of the first 5 min.; BAL was then tipped in from the side-bulbs and a further reading taken over the period 10–30 min. It will be seen that an appreciable amount of reactivation is obtained even under the very adverse conditions of these experiments in which the enzyme was poisoned for periods of up to 26 min. before the addition of BAL.

Tartar emetic is only about one-fourth as toxic to the *brei* as to the dispersion, a relation which was also found with the arsenicals (Stocken *et al.* 1947). The correspondingly high concentrations of BAL which have thus to be used in experiments with the former preparation are themselves appreciably toxic and therefore render the results of the experiments difficult of interpretation. This is a trouble which was also encountered with the arsphenamines (Stocken *et al.* 1947), and is illustrated in Table 3.

Table 1. *The effect of antimonials and BAL on pigeon brain pyruvate oxidase dispersions*

Concentration Sb (mM)	Mol. ratio BAL/Sb	μl. O_2/30 min./2 ml.				Inhibition (%)	
		Control	Sb	BAL	Sb + BAL	Sb	Sb + BAL
				Tartar emetic			
0·05	6	317	119	468	451	62	4
0·05	6	290	112	460	450	61	2
0·05	6	281	113	495	434	60	12
				Stibophen			
0·2	6	456	184	470	470	59	0
0·2	6	334	171	441	452	49	0
0·2	6	468	175	489	488	63	0
				Stibacetin			
0·5	—	406	324	—	—	19	—
1·0	—	406	267	—	—	34	—
2·0	—	408	313	—	—	23	—

Table 2. *The reactivation of brain pyruvate oxidase dispersions by BAL after poisoning with 0·05 mM-tartar emetic*

(*a*, O_2 uptake during 0–5 min.; *b*, during 10–30 min.)

Mol. ratio BAL/Sb	Time of poisoning before addition of BAL (min.)	μl. O_2 ml./2 ml. Control *a*	Control *b*	Sb *a*	Sb *b*	BAL *b*	Sb+BAL *b*	Inhibition (%) Sb *a*	Sb *b*	Sb+BAL *b*
12	24	115	205	42	46	317	198	64	78	38
12	26	110	206	39	59	236	122	65	71	48
12	17	121	193	44	69	281	215	64	64	24

Table 3. *The effect of 0·2 mM-tartar emetic and BAL on pigeon brain* brei *pyruvate oxidase*

Mol. ratio BAL/Sb	0–30 min. inhibition (%) BAL	Sb	Sb+BAL	30–60 min. inhibition (%) BAL	Sb	Sb+BAL
3	0	57	39	18	70	57
3	2	55	59	24	44	66
6	5	55	11	36	44	35
6	8	48	19	40	63	46

It will be seen that there is considerable (18–40 %) inhibition by the BAL alone during the second half-hour period. Nevertheless, in the first half-hour, which is uncomplicated by serious toxic effects due to BAL, it will be seen that some degree of protection is afforded by 6 molecular proportions of BAL.

A few preliminary experiments have been carried out to determine whether injection of BAL can cause the survival of rats poisoned by intramuscular injection of lethal doses of tartar emetic; although evidence has been obtained that BAL exerts significant antidotal properties against antimony under these *in vivo* conditions, further work along these lines is in progress.

Gold. The gold compounds selected were gold chloride (the double salt $AuCl_3.NaCl.2H_2O$), 'myocrisin' (sodium aurothiomalate, hemihydrate) and 1-aurothioglucose (as the double salt with sodium thioglucose).

The results of enzyme experiments with these compounds using the brain dispersion are shown in Table 4.

Table 4. *The effect of gold and thiols on pigeon brain pyruvate oxidase dispersions*

Concentration gold (mM)	Mol. ratio thiol/gold	μl. O_2/2 ml./30 min. Control	Gold	Thiol	Gold+thiol	Inhibition (%) Gold	Gold+thiol
			Gold chloride				
0·017	—	290	250	—	—	14	—
0·085	—	290	193	—	—	33	—
0·085	—	306	213	—	—	31	—
0·17	—	306	119	—	—	61	—
0·43	—	290	26	—	—	91	—
0·43	—	306	31	—	—	90	—
			Gold chloride + BAL				
0·17	3	270	129	378	375	52	1
0·17	3	214	69	395	402	66	0
0·17	3	327	81	455	479	75	0
			Gold chloride + glutathione				
0·17	6	264	140	319	203	47	36
0·17	6	225	55	233	161	76	31
0·17	6	279	144	358	291	48	19
			Myocrisin + BAL				
1·0	3	310	122	260	414	61	0
1·0	3	416	126	270	420	70	0
1·0	3	333	150	242	423	55	0
			Aurothioglucose				
0·5	—	469	388	—	—	17	—
1·0	—	469	371	—	—	21	—
2·0	—	402	273	—	—	32	—
5·0	—	402	247	—	—	39	—
10·0	—	402	186	—	—	54	—

It will be seen that BAL effectively detoxicates both gold chloride and myocrisin. In order to compare a monothiol with the dithiol protection afforded by BAL three experiments have been done using glutathione (Roche Products Ltd.). Although some protection is provided by glutathione it is less active than BAL in this respect.

It will be noted that myocrisin, in which the gold is already attached to a sulphur atom, possesses only approximately one-sixth the toxicity of gold chloride to the pyruvate oxidase system. Aurothioglucose, which possesses in addition to the sulphur combined with gold a molecule of sodium thioglucose, is even less toxic, ten times the concentration being required to produce the same enzyme inhibition as is produced by the presence of 1 mM-myocrisin. For this reason detoxication experiments were not carried out, owing to the high concentrations of BAL which would have been required.

Mercury. In view of the existing evidence that mercury combines with essential —SH groups in enzymes (Rapkine, 1931; Hellermann, 1937; Eagle, 1939; Fildes, 1940) it was of interest to investigate the effect of BAL on the toxicity of mercuric chloride by means of the pigeon brain *brei* preparation.

Fig. 1 shows the effect of increasing concentrations of mercuric chloride in this system, while in Table 5 are given the results of the protection experiments.

It will be seen that 6 molecular proportions of BAL are able to protect the enzyme system completely from the effects of 0·18 mM-mercuric chloride, a concentration which produces approximately 50% inhibition of the unprotected enzyme; the presence of only 3 molecular equivalents is able to cause a very significant degree of protection. Since Fildes (1940) had obtained efficient neutralization of the inhibitory effects of mercuric chloride on the growth of *Esch. coli* by the presence of monothiols such as glutathione or cysteine, enzyme experiments were also done with two representative monothiols, glutathione and 2-mercaptoethanol; the former was found to bring about complete protection, while the latter was completely ineffective under our conditions.

DISCUSSION

The ability of BAL to protect the brain pyruvate oxidase system from poisoning by compounds of arsenic has been shown to hold also for the poisoning produced by compounds of antimony, gold and mercury. In the case of these latter metals, however, the difference in effectiveness, as between dithiols and monothiols, that was shown to hold in the case of poisoning by lewisite (Stocken & Thompson, 1946) does not appear to be so striking. On the evidence so far obtained with gold chloride, glutathione can cause some degree of protection, although it is much less effective than BAL when present in equivalent concentrations. Against mercury, on the other hand, it appears to be an efficient antidote, and Barron (1945) has claimed that it is partially able to reverse the toxic effect of *p*-chloromercuribenzoic acid on the pyruvate oxidase system of gonococci.

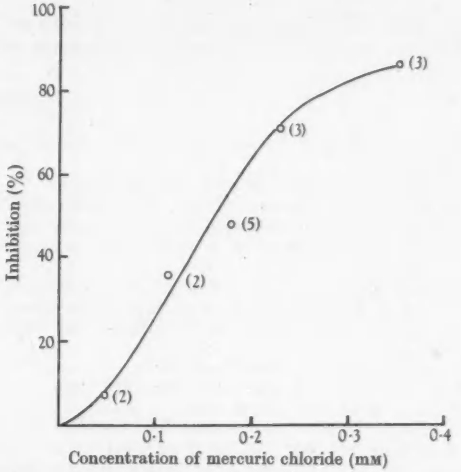

Fig. 1. The inhibition of pigeon brain *brei* pyruvate oxidase by mercuric chloride (no. of experiments averaged is given in brackets).

Table 5. *The effect of thiols on the poisoning of the pigeon brain* brei *pyruvate oxidase system by* 0·18 *mM-mercuric chloride*

Mol. ratio thiol/$HgCl_2$	μl. O_2/g./hr. over 1 hr.				Inhibition (%)	
	Control	$HgCl_2$	Thiol	Thiol + $HgCl_2$	Hg	Hg + thiol
			BAL			
3	2190	1088	2360	2060	51	13
3	1805	1119	1938	1888	38	3
6	2190	1088	1959	2414	51	0
6	1805	1119	1615	1648	38	0
			Glutathione			
12	1979	834	1757	1995	58	0
12	2093	1006	2130	2133	52	0
			2-Mercaptoethanol			
12	1979	834	2035	537	58	74
12	2093	1006	2053	665	52	68

Although insufficient experiments have been carried out to explain the remarkable difference in the effectiveness of glutathione and of 2-mercaptoethanol against mercury inhibition, it is certain that there are many factors which influence the effectiveness of thiol antidotes, among them the relative

stability of the 'tissue acceptor' and antidote-mercaptides, and the relative ease of penetration of inhibitor and antidote to the active centres.

The increased oxygen uptake of brain dispersions brought about by the presence of BAL has been observed before, and has been shown to be due to a true stimulation of the enzyme activity (Stocken *et al.* 1947).

SUMMARY

The ability of 2:3-dimercaptopropanol (British Anti-Lewisite or 'BAL') to detoxicate compounds of antimony, gold and mercury has been demonstrated, using the pyruvate oxidase system of pigeon brain as a test system.

This work was carried out for the Ministry of Supply as part of a larger programme under the direction of Prof. R. A. Peters. Our thanks are due to Dr L. A. Stocken for his helpful suggestions in the course of this work, to the Chief Scientific Officer, Ministry of Supply, for permission to publish, to Messrs May and Baker for a sample of myocrisin, and to the Wellcome Bureau of Scientific Research for samples of stibophen, stibacetin and aurothioglucose.

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The Inhibition of Succinoxidase by Heavy Metals and its Reactivation with Dithiols*

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(*Received* 21 *March* 1946)

Many heavy metals either combine with —SH groups to form mercaptides or act as oxidizing agents. Their combination with the —SH groups of those enzymes requiring the presence of these groups for activity results in enzyme inhibition. Since a large number of enzymes are sulphydryl enzymes (Barron & Singer, 1945; Singer & Barron, 1945), it is reasonable to assume that the toxicity of these heavy metals must be largely due to inhibition of —SH enzymes. Mercaptides are in general easily dissociable, i.e. the metals leave the —SH groups to which they were originally attached when in the presence of other —SH groups with which they form more stable compounds. A typical example of such reactions is that given by Hellerman (1937) when he introduced *p*-chloromercuribenzoic acid (Hg-Cl-benzoate) as an inhibitor of —SH enzymes; the inhibitions produced by it were easily reversed on addition of thiols (cysteine or glutathione). In spite, however, of the knowledge that some heavy metals form mercaptides, very little has been done to see if they combine with the —SH groups of proteins. If

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

such combinations were found to exist, they would explain the mechanism of heavy metal intoxication. Furthermore, it would then be possible to search for the appropriate thiol compounds able to dissociate the enzyme-heavy metal complex.

Of the sulphydryl enzymes, succinoxidase was chosen as a test system because of its easy preparation and reproducible results. The effects produced by those heavy metals expected to combine with the —SH groups of the enzyme were studied and reactivations of the inhibited enzyme with different thiols were attempted.

EXPERIMENTAL

Enzyme preparation. Pigeon breast muscle was used as source of the enzyme. The chopped muscle was poured into a Waring blendor and shaken twice in 200 ml. of water plus 200 g. of cracked ice for 3 min. After each washing the mixture was centrifuged for 30 min. at 3000 r.p.m. and the supernatant fluid was discarded. An equal volume of 0·1 M-Na_2HPO_4 was added to the remaining solid portion and the whole was shaken for 2 hr. at 27°. After centrifugation for 40 min., the supernatant fluid was used for the experiments. This colloidal suspension showed no O_2 uptake in the absence of succinate; in the presence of succinate (0·03 M) it gave a Q_{O_2} value of about 350 (μl. O_2/mg./hr.). The suspension was then diluted so that 0·6 ml. of the diluted suspension showed about 200 μl. O_2 uptake in 30 min. at 38° at pH 7·0. The heavy metals were added in aqueous solutions and kept in contact with the enzyme for 20 min. previous to the measurement of the O_2 uptake. In reactivation studies the thiols were added, as a rule, after the 20 min. of contact between enzyme and heavy metal.

Mercury. Mercury, in the form of an organic mercurial preparation (*p*-chloromercuribenzoate), was introduced by Hellerman (1937) as a mercaptide-forming reagent, the reaction being:

$$R\text{SH} + \text{Hg Cl-benzoate} = R\text{S-Hg-benzoate} + \text{HCl}.$$

Mercuric chloride is extremely toxic to the animal body and has long been considered toxic to enzymes, but the mechanism of its action has not been studied. Haarmann (1943) believes that inorganic Hg is not specific for —SH groups and maintains that it combines primarily with the peptide groups in proteins and secondarily with the N of the imidazole group. In Fig. 1 can be seen the effect of $HgCl_2$ on the activity of succinoxidase where, on plotting concentration of inhibitor against percentage inhibition a typical S-shaped curve was obtained. Hg^{++} at concentrations *c.* 10^{-5} M inhibited succinoxidase, half inhibition being obtained at $1{\cdot}25 \times 10^{-5}$ M concentration of Hg. If Hg^{++}, like the organic mercurial Hg Cl-benzoate, forms reversible mercaptides with —SH groups of proteins, it will be possible to reactivate the enzyme on addition of thiol compounds at the concentration necessary to dissociate the Hg from the protein —SH groups. Effective reversals of Hg inhibition were obtained on addition of BAL and the dithiols 2, 3, 10 and 18 (for nomenclature see Barron, Miller & Kalnitsky (1947)). At a ratio of $HgCl_2$: dithiol compound (in terms of molar concentration) of 1 : 10, dithiols 3, 2 and 10 reactivated the enzyme 70–85 %. BAL produced a reactivation of 63 %, and glutathione (at equivalent SH concentration) of only 21·5 %. When the ratio was

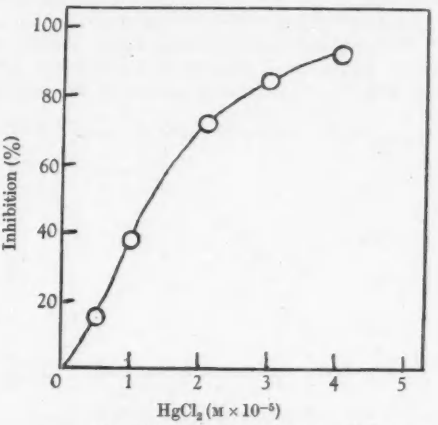

Fig. 1. Inhibition of succinoxidase by $HgCl_2$.

diminished to 1 : 5, dithiols 2, 3 and 10 were still effective reversal agents while the reversal effect of BAL and dithiol 18 diminished to half, i.e. 30 % reactivation. At a ratio of 1 : 3·3 and 1 : 2·2 the dithiols 2 and 3 were still effective (Table 1). The choice reversal agents are therefore dithiols 2, 3 and possibly 10 and their trial in the treatment of $HgCl_2$ poisoning seems warranted. Dr Longcope (Longcope & Luetscher, 1946), has used BAL successfully in the treatment of Hg poisoning.

Table 1. *Reactivation by dithiols of succinoxidase inhibited with* $HgCl_2$

(Original inhibition of enzyme activity, 87·6 %. Enzyme, 0·6 ml.; phosphate buffer, 0·05 M, pH 7·0; succinate, 0·02 M; $HgCl_2$, 3×10^{-5} M; dithiols at concentrations 10, 5, 3·3 and 2·2 times the concentration of $HgCl_2$ added 15 min. after addition of Hg. Vol. 3 ml.; temp. 38°; duration of exp. 30 min.)

	Reactivation (%) at ratios of Hg : dithiol			
Dithiol*	1 : 10	1 : 5	1 : 3·3	1 : 2·2
2	70·0	60·2	46·0	25·5
3	85·8	63·2	42·2	15·0
1	62·7	32·7	0	0
21	66·0	—	—	—
18	67·4	27·3	16·3	5·1
10	81·0	64·6	29·4	6·3
16	44·6	—	—	—
Glutathione (ratio 1 : 20)	21·5	—	—	—

* For this numbering, see Barron, Miller & Kalnitsky (1947); also Glossary, p. 351.

Bismuth. The insolubility of inorganic bismuth salts, and the use of the soluble organic compound NaK-bismuthyl tartrate for therapeutic purposes prompted us to use this compound in our work on the inhibiting effect of this metal. The compound contains 41 % Bi, but its exact structural formula is unknown. A concentration of Bi of approximately 10^{-5} g.-atoms/l. was sufficient to produce inhibitions of succinoxidase, half inhibition being reached at $1{\cdot}2 \times 10^{-5}$ g.-atoms/l. (Fig. 2). This inhibition, like that of Hg, is a reversible inhibition and presumably

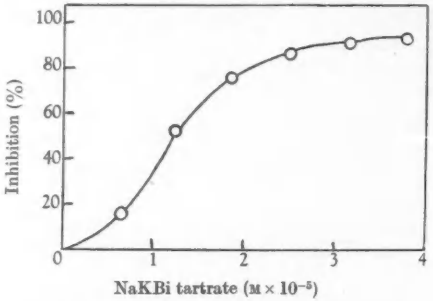

Fig. 2. Inhibition of succinoxidase by bismuth.

due to mercaptide formation. Complete reactivation of the enzyme was attained with dithiols 2 and 3 at a ratio of Bi : dithiol of 1 : 4. At the same ratio, dithiol 10 produced a reactivation of 87 %; BAL

Table 2. *Reactivation by dithiols of succinoxidase inhibited with bismuth*

(Original inhibition by bismuth, 86·5 %. Enzyme, 0·6 ml.; succinate, 0·02 M; phosphate buffer, 0·05 M, pH 7·0; Bi (in form of NaKBi tartrate), $2{\cdot}5 \times 10^{-5}$ M; total vol. 3·0 ml.; duration 25 min.; temp. 38°; duration of exp. 30 min. Dithiol added 20 min. after addition of Bi.)

	Reactivation (%) at ratios of Bi : dithiol					
Dithiol	1 : 10	1 : 4	1 : 3·2	1 : 1·6	1 : 0·8	1 : 0·4
2	—	100	92·5	66·3	27·6	—
3	—	100	100	78·2	47·8	37·9
1	—	45·3	25·5	19·8	—	—
21	72·4	—	—	—	—	—
18	—	14·9	2·1	1·0	—	—
10	—	86·9	58·4	23·1	30·6	0·0
16	56·4	—	24·4	—	—	—

reversed the inhibition by only 45 %. When the concentrations of dithiols were reduced to a ratio of Bi : dithiol of 1 : 0·8, dithiol 3 was able to produce half-reactivation (Table 2). The affinity of this dithiol for Bi is so great that its use in the treatment of Bi poisoning is strongly recommended.

Cadmium. It was shown in this laboratory in collaboration with Dr Gerard's co-workers that cadmium is an SH enzyme inhibitor, and Tobias, Lushbaugh, Patt, Postel, Swift & Gerard (1946) have shown that mice poisoned with this metal can be successfully treated by the administration of BAL. Although it has been reported that metallic Cd and CdO are more toxic than $CdCl_2$ (Irwin & Semmons, 1946) this last salt was used in these experiments. Enzyme inhibition was obtained at concentrations around 10^{-6} M-Cd, half inhibition requiring 7×10^{-6} M (Fig. 3). Dithiols 2, 3 and 10

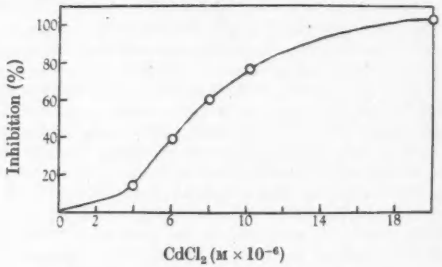

Fig. 3. Inhibition of succinoxidase by $CdCl_2$.

were more effective than BAL in producing reactivation of the enzyme. Thus, at a ratio of Cd : dithiol of 1 : 10 there was almost complete reactivation with dithiol 3 while BAL produced only half-reactivation. At this ratio, glutathione produced almost no reactivation. When the ratio was diminished to 1 : 1, dithiol 3 was able to produce half-reactivation while BAL had no effect at all (Table 3). It is possible to obtain complete reactivation of the enzyme with glutathione when the ratio Cd : SH compound is increased to 1 : 500.

Table 3. *Reactivation by dithiols of succinoxidase inhibited with cadmium*

(Original inhibition by Cd, 80·7 %. Enzyme, 0·6 ml.; succinate, 0·02 M; phosphate buffer, 0·05 M, pH 7·0; $CdCl_2$, 1×10^{-5} M; total vol. 3·0 ml.; temp. 38°; duration of exp. 30 min. Dithiol added 20 min. after addition of Cd.)

	Reactivation (%) at ratios of Cd : dithiol			
Dithiol	1 : 20	1 : 10	1 : 2	1 : 1
2	—	81·6	64·8	14·6
3	—	92·2	55·1	49·4
1	—	57·8	20·5	0·0
21	—	65·4	—	—
18	—	15·7	0·0	0·0
10	—	78·2	59·2	10·2
16	—	19·8	—	—

Vanadium. Vanadium in the form of VCl_2 produced inhibition of succinoxidase at concentrations around 10^{-4} M. Half inhibition of enzyme activity

was obtained with $1{\cdot}1 \times 10^{-4}$ M (Fig. 4). Reversal of vanadium inhibition was obtained only on addition of dithiol 3, a reversal which was obtained even at a ratio of V : dithiol of 1 : 2·2. At this ratio, dithiol 3 produced a reactivation of 79%; dithiol 2 reactivated the enzyme by only 9%; while the remaining dithiols, including BAL, had no effect at all on the vanadium inhibition of succinoxidase (Table 4).

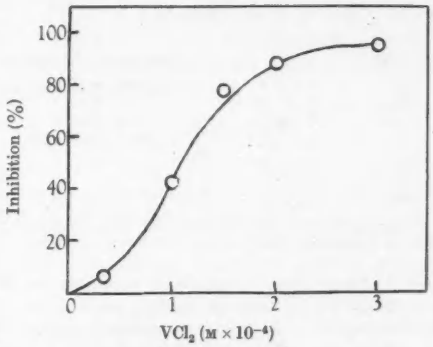

Fig. 4. Inhibition of succinoxidase by vanadium.

Table 4. *Reactivation by dithiols of succinoxidase inhibited with vanadium*

(Original inhibition by VCl_2, 78·2%. Enzyme, 0·6 ml.; succinate, 0·02 M; phosphate buffer, 0·05 M, pH 7·0; VCl_2, $1{\cdot}5 \times 10^{-4}$ M; vol. 3·0 ml.; temp. 38° Dithiol added 20 min. after addition of V to enzyme.)

	Reactivation (%) at ratios of V : dithiol		
Dithiols	1 : 8·5	1 : 7	1 : 2·2
2	—	16·6	9·0
3	80·0	78·0	79·0
1	—	9·0	0
18	—	0	0
10	—	12·0	3·0

Selenium. Collett (1924, 1933) found that selenium and tellurium inhibit the activity of succinoxidase. This inhibition is undoubtedly due to their action on the —SH groups of the activating protein. Selenium also inhibits the activity of another —SH enzyme, papain (Bersin & Logerman, 1933). The inhibiting effect of selenite was quite different from that produced by heavy metals. In fact, concentrations of selenite *c.* 10^{-5} M produced a slight inhibition of the enzyme, which did not increase on addition of increasing amounts of selenite until a concentration of 10^{-4} M was reached. Around this concentration the inhibition rose with the concentration of inhibitor. Half inhibition was obtained at $1{\cdot}1 \times 10^{-4}$ M-selenite (Fig. 5). Reversal of inhibition was not as effective as with heavy metal inhibition. Dithiols 2 and 3 at a ratio of Se : dithiol of 1 : 7 produced about half-reactivation, while BAL and other dithiols used had no effect at all (Table 5). A possible explanation of this failure may be due to the large amount of dithiols required for reactivation for we have already shown that dithiols at concentrations above 1×10^{-3} M inhibit succinoxidase.

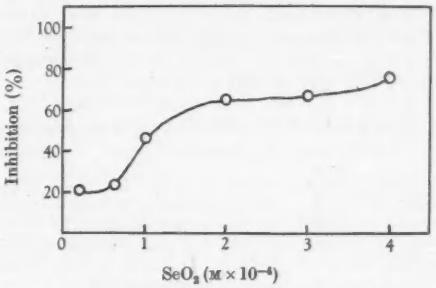

Fig. 5. Inhibition of succinoxidase by SeO_2.

Table 5. *Reactivation by dithiols of succinoxidase inhibited by selenium*

(Original inhibition by SeO_2, 75·4%. Enzyme, 0·6 ml.; succinate, 0·02 M; phosphate buffer, 0·05 M, pH 7·0; SeO_2, 4×10^{-4} M; vol. 3·0 ml.; temp. 38°. Dithiol added 20 min. after addition of Se to enzyme.)

	Reactivation (%) at ratios of Se : dithiol		
Dithiols	1 : 7	1 : 5	1 : 2·3
2	41·4	32·6	0
3	54·3	45·6	15·4
1	0	0	0
18	0	0	0
10	0	10·9	0

Zinc. Zinc inhibition of succinoxidase was obtained only with relatively high concentrations of $ZnCl_2$, half inhibition requiring $1{\cdot}5 \times 10^{-3}$ M-Zn. Reactivations were complete on addition of 0·03 M-glutathione. Gilman & McNamara (1946) have shown that BAL is of value in preventing the development of pulmonary lesions after inhalation of zinc fumes.

Lead. Since Pb is closely related to Hg and Bi by its position in the periodic table, there is a possibility that this metal is also an inhibitor of —SH enzymes. However, because of the insolubility of lead phosphate (the enzyme is extracted by 0·1 M-Na_2HPO_4), the experiments on lead were performed after dialysis of the enzyme in running water and resuspension in acetate buffer. Under these conditions, at pH 6·3, lead inhibited succinoxidase. Half-inhibition was reached at a concentration of $2{\cdot}5 \times 10^{-4}$ M.

Effect of inhibitor concentration on extent of enzyme reactivation. In the experiments reported above, the inhibitor was used at a concentration sufficient to

produce from 75 to 87 % inhibition. When the concentration of the inhibitor is increased so as to produce complete inhibition, reactivation becomes much more difficult, possibly because of irreversible denaturation. This effect was tested with Bi as the heavy metal inhibitor. Up to a certain concentration ($1{\cdot}2 \times 10^{-5}$M-Bi) the inhibition was reversed on addition of dithiol 3 by 75 %; when the concentration was increased ($2{\cdot}5 \times 10^{-5}$M) to produce 95 % inhibition, the reversal on addition of dithiol dropped to only 13·5 % (Table 6).

Table 6. *Reversal of inhibition with increasing concentration of inhibitor*

(Enzyme, 0·6 ml.; succinate, 0·02M; phosphate buffer, 0·05M, pH 7·0; Bi (NaKBi tartrate) in indicated concentrations; dithiol 3, 1·6 times concentration of Bi; vol. 3·0 ml.; temp. 38°. Dithiol added 20 min. after addition of Bi to enzyme.)

Concentration of Bi (M)	Inhibition (%)	Dithiol added. Ratio Bi : dithiol	Reactivation (%)
$1{\cdot}2 \times 10^{-5}$	87·0	1 : 1·6	74·7
$1{\cdot}9 \times 10^{-5}$	88·0	1 : 1·6	28·0
$2{\cdot}5 \times 10^{-5}$	95·3	1 : 1·6	13·5

Effect of time on enzyme reactivation. It has been pointed out that the protein component of —SH enzymes tends to be irreversibly denatured when the —SH groups have undergone chemical change. In order to determine the minimum time for complete reactivation of —SH enzymes (in this case succinoxidase) after addition of heavy metal inhibitors, experiments were performed where the selected dithiols were added at different intervals of time after addition of the heavy metal (Table 7).

Table 7. *Effect of addition of dithiol at various times after contact of enzyme with bismuth*

(Enzyme, 0·6 ml.; succinate, 0·02M; phosphate buffer, 0·05M, pH 7·0; Bi (in form of NaKBi tartrate) in indicated concentrations; concentration of dithiol 3, 1·6 times concentration of Bi; temp. 38°.)

Concentration of Bi (M)	Reactivation (%) on addition of dithiol at various times (min.) after contact of enzyme with Bi: 0	15	20	30	45	60
$3{\cdot}1 \times 10^{-5}$	—	45	—	34	27	2·7
$2{\cdot}5 \times 10^{-5}$	87·6	—	87·6	76·8	74·5	—

On addition of heavy metal in sufficient quantity to produce 88 % inhibition ($2{\cdot}5 \times 10^{-5}$M-Bi) the enzyme was effectively reactivated (88 %) on addition of dithiol 3 at a ratio of 1 : 1·6, 20 min. after Bi addition. After 45 min., there was still good reversal (74 %). When the concentration of Bi was raised to $3{\cdot}1 \times 10^{-5}$M-Bi, addition of dithiol 30 min. after metal addition produced a reactivation of only 34 %; the extent of reactivation decreased to 27 % when the dithiol was added 45 min. after the Bi. At the end of 1 hr. the enzyme had been irreversibly inhibited.

Table 8. *Comparison of toxicity of heavy metals for succinoxidase system*

(Succinoxidase, 0·6 ml.; succinate, 0·01M; phosphate-HCl buffer, 0·033M, pH 7·0; metals in indicated concentrations. Total vol. 3·0 ml.; temp. 38°.)

Metal inhibitor	Half-inhibition (M)	Best reversal agent*
Bi	$1{\cdot}2 \times 10^{-5}$	3, 2, 10, BAL
Hg	$1{\cdot}25 \times 10^{-5}$	3, 3, 10, BAL
Cd	7×10^{-6}	3, 2, 10, BAL
V	$1{\cdot}1 \times 10^{-4}$	3, BAL ineffective
Se	$1{\cdot}1 \times 10^{-4}$	2=3
Zn	$1{\cdot}5 \times 10^{-3}$	—
Sb	$2{\cdot}0 \times 10^{-3}$	—

* Numbers refer to thiols listed in the glossary of Barron, Miller & Kalnitsky (1947).

In Table 8 there is given a summary of the toxicity of heavy metals studied as well as the dithiols found to be the best reversal agents. It must be emphasized that in all cases of heavy metal inhibition BAL was not as effective as dithiols 2 and 3. It may be noted that Dr Harry Eagle (personal communication) has reported that BAL was not effective in the treatment of Bi intoxication produced in rabbits.

Antimony. To study the effect of this metal on the activity of succinoxidase the organic antimony compound Fuadin* (sodium salt of sodium antimony *bis*catechol disulphonate) was used. According to the distributors of this drug 1 ml. of this solution contains 8·5 mg. of antimony. The reducing substances contained in the solution were oxidized with 0·1N-iodine. Under those conditions the aqueous solutions of Fuadin inhibited the activity of succinoxidase; 2×10^{-3}M-Sb produced half inhibition. The inhibition was reversed on addition of 4×10^{-2}M-glutathione. Attempts to reactivate the enzyme with BAL or dithiol 2 were unsuccessful because of the relatively large concentration of dithiol theoretically required to achieve reactivation.

DISCUSSION

It is very difficult to decide without further experimentation the kind of linkage formed between the heavy metal and the —SH groups of the protein molecule and to give any quantitative treatment to the data on degree of inhibition with increasing concentrations of metal. The heavy metal (M) may combine with two —SH groups: Protein M

* Fuadin was generously donated by Winthrop and Company.

forming cyclic compounds; it may combine with only one —SH group: Protein-S-MR', as seems to happen with the organic mercurial complexes. If only the first reaction occurs, the dissociation constant of the reaction between the —SH groups of the protein and the metal would be obtained from equation

$$K=\frac{(\text{—SH})^2\,(M)}{\left(M\begin{smallmatrix}\text{S—}\\ \text{S—}\end{smallmatrix}\right)}.$$

The second type of compound would give rise to the dissociation constant

$$K=\frac{(\text{—SH})\,(MR)}{(M\text{—S—}R')}.$$

If both reactions took place, the two equations would have to be extended and the contributions of one or the other would be difficult to determine.

There is, however, indirect evidence in favour of the opinion that heavy metals might combine with the —SH groups of the protein, forming metal cyclic compounds. This evidence is given by the rapid reactivation of heavy metal inhibition on addition of dithiols while monothiols (glutathione) were poor agents for reactivation.

Selenite possibly acts as an oxidizing agent. This would explain the lack of complete reactivation on addition of dithiols, the partial reactivation being possibly due to the reducing power of these compounds. However, it is possible that Se might form the compound R—S—Se—S—R (Painter, 1941).

In conclusion we must make a plea for studying in more detail the therapeutic properties of the dithiols. BAL has proved to be an efficient agent for the treatment of lewisite poisoning and Rhoads and co-workers have reported that dithiol 9 is more efficient than BAL for the treatment of arsine poisoning. The work here presented is an indication that the dithiols 2 and 3 might be more efficient than BAL in the treatment of heavy metal poisoning.

SUMMARY

1. A large number of heavy metals has been found to inhibit the enzyme activity of succinoxidase. The inhibition is due to combination of the metal with the —SH groups of the protein moiety. Bi, Cd and $HgCl_2$ had the greatest inhibitory power, producing 90 % inhibition at *c.* 10^{-5} M concentration. V, Zn and Pb produced effective inhibitions at about 10^{-4} M. Zn and Sb required higher concentrations, half-inhibition being attained at about $1{\cdot}5\times10^{-3}$ M.

2. The inhibition produced by these heavy metals was reversed on addition of dithiols. The most effective compounds for the reversal of Bi, Hg and Cd inhibition were 1:3-propanedithiol, 1:3-dimercaptopropanol and 2:3-dimercaptopropionic acid. 1:3-Propanedithiol was the only effective agent for the reversal of V inhibition. 1:3-Dimercaptopropanol and 1:3-propanedithiol were effective against Se inhibition. Reversal by BAL (2:3-dimercaptopropanol) occurred only at higher concentrations. Glutathione at the concentrations used with dithiols was generally ineffective; at higher concentrations it produced reactivation.

3. Reversal of inhibition became increasingly difficult when the concentration of inhibitor was raised so as to produce complete inhibition.

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GLOSSARY

Compound	Name	Compound	Name
1	2:3-Dimercaptopropanol (BAL)	10	2:3-Dimercaptopropionic acid
2	1:3-Dimercaptopropanol	16	bis-*S*(*N*-Ethylacetamidomethyl) ether of BAL
3	Propane-1:3-dithiol	18	2:3-Dimercaptopropyl propionate
9	2:3-Dimercaptopropyl ethyl ether	21	3:4-Dimercaptobutanol-1

The Fate of Arsenical Vesicants in the Skin and the Effect of BAL (British Anti-Lewisite)

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The work described in this paper was carried out on behalf of the Ministry of Supply, and preliminary accounts of it were circulated by the Ministry in 1943 and early in 1944. A quantitative investigation was made of various factors concerned in the disappearance of arsenic from the skin after the application of an arsenical vesicant. These factors determine the severity of the local and systemic effects caused by a given amount of a vesicant. Phenyldichloroarsine and lewisite I (β-chlorovinyldichloroarsine) were studied, using rabbits as experimental animals. The amount of arsenic removed from the skin by absorption into the general circulation or evaporation of the vesicant, and the fraction of the residual arsenic which was fixed by combination with skin constituents, were determined at varying periods after applying the vesicant. The effect of varying the amount of vesicant applied to a given area of skin was examined. Stocken & Thompson (1946) had previously found that BAL (British Anti-Lewisite, 2:3-dimercaptopropanol) diminished the amount of arsenic remaining in the skin of rats 48 hr. after contamination with lewisite. Experiments were done to discover the way in which BAL modifies the fate of arsenical vesicants in the skin.

EXPERIMENTAL

General methods

The vesicants (obtained from the Ministry of Supply) were purified by distillation *in vacuo*. They were applied to the skin as freely falling drops, measured from the 'burette' devised by Lane (1946) or from the modification of this instrument developed by Levvy (1946). The amounts of liquid to be measured were so very small that they could be delivered accurately only in the form of discrete drops. In using Lane's instrument, it was found convenient to retard the drop rate by applying a negative hydrostatic pressure to the column of vesicant. In experiments with phenyldichloroarsine, two different drop sizes were employed (5 and 0·5 mg.), and in the case of lewisite I, three (4·5, 0·5 and 0·133 mg.). The exact weight of the drop applied in an individual experiment was calculated from the readings given by the instrument at the time, and this figure was used in working out the results. For drops of any one magnitude, the range from experiment to experiment in the calculated figure for the weight was not more than 5% of the mean.

The rabbits used ranged in weight from 2 to 3 kg. and were of both sexes. For treatment with the vesicant, an area of skin on the back was freed from fur by clipping closely with scissors. One or more circles, approximately 3 cm. in diameter, were drawn in Indian ink on the skin, as far apart as possible if there were more than one. One drop of vesicant was allowed to fall on to each of these marked out 'spots'. In some cases the spot was immediately covered with a glass vessel, 2 cm. in diam. and 1 cm. in depth, which was firmly attached to the skin with adhesive plaster. Open glass cylinders of the same dimensions were secured to spots which were to be left uncovered so as to prevent the animal from removing the vesicant. The animal was kept in a warm room for a predetermined period and then killed. A piece of skin of 1·5–2 cm. radius around the point of impact of the drop of vesicant was excised, all underlying fatty tissue being left behind.

The excised skin was analyzed directly for arsenic or submitted to preliminary extraction with water or acetone. When arsenic was to be directly determined, the glass cover or guard was washed with benzene and arsenic in the washings was estimated separately or together with that in the skin, after removal of the benzene in presence of water. No appreciable amounts of arsenic were ever found in the benzene washings when analyzed separately. Extractions were carried out in a 50 or 100 ml. Soxhlet apparatus, the skin (plus glass cover or guard) being placed directly in the extraction chamber since paper thimbles were found to adsorb some of the arsenic. Arsenic was determined in the extract and residue, after the removal of acetone if it was initially present.

Portions of the extracts were dialyzed through 'Visking' sausage casing in the refrigerator. To prepare acetone extracts for dialysis, this solvent was replaced by evaporation *in vacuo* with at least 2 vol. water. Although this process resulted in the formation of a fat emulsion, the amount of arsenic in solution did not depart from the original figure.

Arsenic was determined by Levvy's method (1943), using the small-scale digestion procedure. Whenever the amount of arsenic permitted, three or more portions of the skin digest or extract were analyzed. Control experiments were frequently done in which the arsenic in normal skin or filter paper treated with a drop of the vesicant was determined. This gave a check on the measurement of the vesicant and on the analytical procedure. Satisfactory results were obtained throughout. Normal rabbit skin was found to contain no detectable amounts of arsenic.

RESULTS

The absorption of vesicants from the skin

In these experiments the contaminated skin was covered to prevent loss of vesicant by evaporation. The absorption into the general circulation in a given period was found by determining the total residual arsenic. Results for the absorption of lewisite I are shown in Table 1 and of phenyldichloroarsine in the average amount of arsenic removed being about 45 % of the total applied.

Table 1. *The disappearance of lewisite I from covered skin*

Time after application (hr.)	No. of experiments done	Average for arsenic absorbed as % applied
	4·5 mg. drops	
0·25	1	21
0·5	2	21
1	2	24
2	3	37
3	1	58
4	3	56
6	1	76
10	1	72
16	2	83
24	3	78
30	1	89
48	2	90
96	1	91
168	1	85
	0·5 mg. drops	
0·25	3	27
0·5	2	36
1	1	59
2	1	44
4	2	55
6	1	60
24	1	51
48	1	73
96	1	61
	0·133 mg. drops	
1	1	40
24	1	48
72	1	50

Table 2. Some of the figures represent results for experiments with single animals whilst others are the averages for several animals. Since the rate of absorption of either vesicant appeared to be independent of the total number of drops applied, this factor has been ignored in compiling the tables. In most of the experiments the animal was treated with the vesicant at two points, in a few at one only and in a limited number at more than two.

It can be seen from Table 1 that absorption from 4·5 mg. drops of lewisite I ceased after about 24 hr., by which time 90 % had been transported from the skin; with 0·5 mg. drops, 60 % was absorbed in 1 hr. and there appeared to be little change thereafter. Absorption from 0·133 mg. drops showed little variation over the time range studied (1–72 hr.),

After 24 hr., the absorption of phenyldichloroarsine (Table 2) was about 45 % from 5 mg. drops and 35 % from 0·5 mg. drops. The divergence of the results for the two drop sizes can be accounted for from the initial delay of ½ hr. before absorption from the smaller commenced. With both drop sizes, absorption, although slow, still appeared to be in progress after 6 or 7 days. Absorption of phenyldichloroarsine was not only slower than that of lewisite I, but in contrast to the latter never appeared to reach completion in the period studied.

With either vesicant, absorption must be influenced to a great extent by the rate of hydrolysis on and in the skin to the less penetrant arsenoxide.

Table 2. *The disappearance of phenyldichloroarsine from covered skin*

Time after application (hr.)	No. of experiments done	Average for arsenic absorbed as % applied
	5 mg. drops	
0·5	3	13
1	3	9
1·5	1	12
2	1	21
3	1	22
4	6	32
6	1	39
10	2	21
18	1	40
24	3	43
30	1	50
48	1	49
96	1	47
168	1	56
	0·5 mg. drops	
0·5	2	0
1	1	17
2	1	18
3	1	20
4	2	25
6	1	21
15	1	29
24	3	35
48	1	31
96	2	33
144	1	54

While hydrolysis of both compounds studied occurs extremely rapidly in water, it is probably greatly delayed in the outer layers of the skin. It seems certain, however, that all the arsenic transported from the skin in the blood will be in the form of the arsenoxide.

The evaporation of vesicants from the skin

It was considered that comparison of the rates of disappearance of arsenic from covered and from uncovered skin should reveal any loss of vesicant by evaporation. Table 3 shows the rate at which

lewisite I disappeared from uncovered skin. Each animal in these experiments was treated with either one or two uncovered drops of vesicant. As before, the number of drops applied to the animal did not affect the result. On comparing the results in Table 3 with those in Table 1, it seems that, if any loss of the vesicant by evaporation did occur, it did not exceed 10 % of the total applied.

Table 3. *The disappearance of lewisite I from uncovered skin*

Time after application (hr.)	No. of experiments done	Average for arsenic lost by absorption and evaporation as % applied
	4·5 mg. drops	
0·5	1	24
1	3	36
2	1	47
4	2	61
6	2	78
10	1	80
24	3	87
48	1	96
96	1	83
168	1	96
	0·5 mg. drops	
0·5	2	59
4	3	67
24	1	79

A slightly different procedure was followed in studying the evaporation of phenyldichloroarsine from the skin. Each animal was treated with equal numbers of covered and uncovered drops of vesicant (usually one of each). The results for removal of arsenic from covered skin in these experiments have already been shown under the appropriate times in Table 2. Table 4 gives the corresponding figures for uncovered skin. Comparison of the two sets of results suggests that up to 25 % of the vesicant applied may evaporate from the skin.

Table 4. *The disappearance of phenyldichloroarsine from uncovered skin*

Time after application (hr.)	No. of experiments done	Average for arsenic lost by absorption and evaporation as % applied
	5 mg. drops	
1	3	18
4	6	55
10	2	48
24	3	49
	0·5 mg. drops	
4	2	42
24	3	50

It should be pointed out that absorption and evaporation are probably mutually dependent processes, each decreasing the amount of vesicant available for removal by the other. Experiments of the type described above can therefore be regarded as of qualitative value only.

To discover whether under ideal conditions evaporation of phenyldichloroarsine from the skin would proceed at all rapidly, dry filter paper was treated with 5 mg. drops and suspended in a current of dry air in an incubator at 38°. Only 60 % of the arsenic disappeared in 4 hr. and 70 % in 24 hr.

The fixation of arsenical vesicants in the skin

Arsenic which could not be extracted by fat solvents was considered to have been fixed by combination with skin constituents. In studying this, each animal was treated with the vesicant in two places. One piece of contaminated skin was extracted with a fat solvent, almost always acetone, while in the majority of experiments the other was extracted with water. All extractions were done for periods found to be more than ample for complete removal of arsenic extractable by the solvent.

Table 5 shows the free (acetone-soluble) and fixed (acetone-insoluble) arsenic in skin at varying periods after the application of lewisite I, while Table 6 gives the corresponding figures for phenyldichloroarsine. Since the fixed arsenic was never more than a small fraction of the total originally present, it is given as μg. arsenic as well as in terms of percentage applied.

Table 5. *The fixation of lewisite I in the skin*

(Single experiments; skin extracted with acetone.)

Time after application (hr.)	Extract as % applied	Residue	
		μg. As	% applied
	4·5 mg. drops, uncovered		
3	31	73	5
6	25	52	3
24	10	101	6
72	4	29	2
	4·5 mg. drops, covered		
0·25	81	88	5
0·5	84	83	5
4	46	198	11
4·5	43	210	13
48	12	191	12
96	18	246	14
	0·5 mg. drops, uncovered		
4	—	20	11
24	5	20	11
	0·5 mg. drops, covered		
0·5	44	24	13
25	12	29	16
	0·133 mg. drops, covered		
1	43	8	16
24	41	11	22
72	8	14	28

With 4·5 mg. drops of lewisite I, 5% of the arsenic applied was fixed at an early stage. When the skin was left uncovered, this figure remained constant for a considerable period. When the skin was covered, however, the arsenic fixed had risen 4 hr. after application to 12% of the total. With 0·5 mg. drops of lewisite I, the arsenic fixed was about 12% of the total applied, and the difference between covered and uncovered skin was less marked than with the larger drops. About 20% of the vesicant was fixed when it was administered as 0·133 mg. drops (covered). It should be noted that free arsenic was still present at periods when on the basis of former experiments absorption must have ceased.

When phenyldichloroarsine was applied to the skin, approximately 4% of the arsenic was fixed with 5 mg. drops (covered and uncovered), and at least 10% with 0·5 mg. drops. As expected from the results for the absorption of this vesicant, considerable amounts of free arsenic were present after 24 hr. On the whole it seems to be safe to say that with both vesicants the fraction fixed in the skin increased as the size of drop applied was made smaller.

Table 6. *The fixation of phenyldichloroarsine in the skin*

(Unless indicated to the contrary, skin extracted with acetone.)

Time after application (hr.)	Extract as % applied	Residue	
		μg. As	% applied
	5 mg. drops, uncovered		
4	60	68	4
24	29	73	5
	5 mg. drops, covered		
0·5	76	26	2
0·5	85	78	5
4	71	75	5
10	59	64	4
10*	47	55	3
24	37	42	3
24†	36	66	4
24	58	92	6
24‡	44	46	3
	0·5 mg. drops, uncovered		
24	9	17	10
	0·5 mg. drops, covered		
1	90	8	5
4	65	18	11
24	60	27	17

* Extracted with benzene instead of acetone.
† Skin desiccated before extraction with acetone.
‡ Extracted with acetone, benzene, ether and carbon tetrachloride.

In one of the experiments with phenyldichloroarsine shown in Table 6, the skin was dried *in vacuo* over H_2SO_4 for 14 days before extraction with acetone. This treatment did not appear to affect the course of the extraction in any way. In other experiments listed in Table 6, the effects of extracting with fat solvents other than acetone were examined. Extraction with benzene took the same course as with acetone. When, after exhaustive treatment with acetone, the skin was extracted successively for 12 hr. with each of the following solvents, the amounts of arsenic in each extract were: acetone, 695; benzene, 5; ether, 6; carbon tetrachloride, 8 μg. As. It thus appears that the behaviour of the arsenic in skin towards extraction by a fat solvent is independent of the particular solvent used. The arsenic in such extracts probably corresponds to the free vesicant and its hydrolysis product.

All the arsenic in skin at any period after treatment with lewisite I or phenyldichloroarsine could be removed by extraction with water. In an experiment with phenyldichloroarsine, the skin after exhaustive extraction with acetone was extracted with water. All the acetone-insoluble arsenic was thereby removed.

The effects of BAL

The effects produced by dropping BAL (obtained from the Ministry of Supply) on to the contaminated skin on the absorption and fixation of lewisite I and phenyldichloroarsine are shown in Table 7. Each animal was treated with the vesicant at two points, both areas of skin being treated with BAL, and the skin was left uncovered in all experiments. Figures in the table for the dose of BAL refer to the amount applied to each contaminated area. After excision, one piece of skin was analyzed for total arsenic while the other was extracted with acetone. The results obtained by direct analysis for the absorption of arsenic are not quoted since they were in good agreement with the corresponding figures calculated from the total arsenic in extract plus residue (last col. in Table 7). It is at once apparent from the table that percutaneous administration of BAL reduced the arsenic fixed with either vesicant to, at the most, only a small fraction of its former value. Up to 6 hr. delay in applying the BAL did not affect the final result, showing that the fixation of arsenic was actually reversed. As regards absorption, comparison with Tables 3 and 4 reveals no appreciable hastening of this process. While the effect of BAL on the fixed arsenic might be expected to decrease the residue found at the end of any given period, the amount fixed is such a small fraction of the total applied that any change produced in the absorption will be masked by the very large animal variation.

In other experiments with lewisite I, BAL was given by injection either before or after applying the vesicant (Table 8). Subcutaneous injections were done about 4 cm. from each contaminated area, the dose shown in the table being divided between the two points of injection. Intravenous injections were made into the ear. All BAL solutions were made up immediately before use. Except in two

Table 7. *The effect of percutaneous administration of BAL on the fate of phenyldichloroarsine and lewisite I in the skin*

(Dose of BAL expressed as amount applied to each contaminated area.)

Time killed after contamination (hr.)	Time of BAL administration after contamination (hr.)	Amount of BAL (mg.)	Extract as % applied	Residue		Arsenic absorbed as % applied
				μg. As	% applied	
		5 mg. drops of phenyldichloroarsine				
24	2	26	51	0	0·0	49
		0·5 mg. drops of phenyldichloroarsine				
24	2	26	30	0	0·0	70
		4·5 mg. drops of lewisite I				
2	0·1	28	55	0	0·0	45
4	0·5	25	45	12	0·7	54
4	2	28	25	0	0·0	75
6	4	28	25	11	0·7	74
7	0·1	28	40	6	0·4	60
7	2	28	42	2	0·1	58
24	0·1	28	15	17	1·0	84
24	0·5	28	33	6	0·3	67
24	2	28	8	4	0·2	92
24	4	28	12	12	0·7	87
96	2	26	11	16	1·0	88
96	6	26	6	5	0·3	94
		0·5 mg. drops of lewisite I				
1	0·1	5	57	0	0·0	43
24	0·1	5	11	1	0·5	88
96	2	26	3	1	0·8	96
96	2	25	6	3	1·4	93
96	6	26	7	3	1·6	91

Table 8. *The effect of injection of BAL on the fate of lewisite I in the skin*

(Dose of BAL expressed as total amount injected into the whole animal. Unless indicated to the contrary, BAL injected subcutaneously in peanut oil.)

Time killed after contamination (hr.)	Time of BAL administration with respect to contamination (hr.)	Amount of BAL (mg.)	Extract as % applied	Residue		Arsenic absorbed as % applied
				μg. As	% applied	
		4·5 mg. drops				
1	−1	200	30	59	4	66
2	−1	100	22	62	4	74
4*	+0·1	60	49	70	4	47
4†	0 and +0·5	50	18	153	9	73
24†	0, +0·5 and +4·5	75	5	127	8	87
96	0	200	2	9	1	97
		0·5 mg. drops				
2	−1	100	38	9	5	57
96	0	100	5	15	8	87

* Subcutaneous injection of BAL in 0·9% NaCl.
† Intravenous injection of BAL in 0·9% NaCl.

experiments in which BAL was given intravenously, the contaminated skin was left uncovered. BAL did not affect the fixed arsenic in these experiments. While the results are not clear on this point, it is doubtful whether absorption was hastened.

Dialysis of water and acetone extracts of skin

In a great number of the experiments with both vesicants, samples of the extracts were submitted to dialysis. In every case, the arsenic in either type of

extract was completely dialyzable. This was also true for acetone extracts in the experiments with BAL.

DISCUSSION

From the results of the experiments described above, it is possible to formulate certain broad principles regarding the fate of lewisite I or phenyldichloroarsine applied to the skin of rabbits. With both vesicants, an increase in the size of drop applied favoured absorption into the general circulation at the expense of the arsenic (free and fixed) remaining at the site of contamination. Absorption of lewisite I rapidly reached a maximum (characteristic of the drop size) and then ceased. The fraction of a given amount of phenyldichloroarsine which was absorbed in 24 hr. was only about half the corresponding figure for lewisite I, but absorption, although very slow, still appeared to be in progress after much longer periods. While the experiments dealing with evaporation were unsatisfactory from a quantitative point of view, it appears that while the amount of lewisite I which volatilized from the skin was so small as to be negligible, appreciable amounts of phenyldichloroarsine did evaporate. A fraction of the arsenic applied could not be removed by extraction with acetone. This fraction was smaller the larger the drop applied, and there was no important difference between the two vesicants in the amounts of arsenic fixed.

The only effect of BAL administration on the arsenic in skin was to prevent or reverse the fixation of arsenic, and this effect was only seen when the BAL was applied to the contaminated area. The rate of absorption of arsenic was not noticeably increased by BAL. These experiments thus provide direct evidence for an *in vivo* reversal by BAL of a reaction between the arsenicals and skin constituents, and it appears likely that the fixed arsenic as measured by its insolubility in acetone is the fraction responsible for the local reaction. It is interesting to note that in unreported work it was found that 1 hr. after intravenous injection of phenylarsenoxide or *m*-amino-*p*-hydroxyphenylarsenoxide into rabbits, none of the arsenic in liver could be extracted with acetone. After 24 hr., up to 30 % of the arsenic was extractable. Subcutaneous injection of BAL decreased the arsenic content, but did not affect the percentage extracted.

The explanation of the finding by Stocken & Thompson (1946) that the arsenic remaining in the skin of rats 48 hr. after treatment with lewisite was decreased by BAL would appear to be that in their experiments practically all the arsenic left in the skin after this period was fixed. In work subsequent to our own it was found by L. Young and his colleagues in Toronto that BAL did not affect the rate of disappearance of arsenic from the skin of rats treated with lewisite, even when applied directly to the contaminated area (private communication).

SUMMARY

1. Lewisite I (β-chlorovinyldichloroarsine) applied to the skin of rabbits was more rapidly absorbed into the general circulation than phenyldichloroarsine.

2. With both vesicants the percentage absorption was greater the larger the amount applied to the skin.

3. Evaporation from the skin appeared to be negligible with lewisite I, but appreciable with phenyldichloroarsine.

4. At any time after applying either vesicant, part of the arsenic in the skin could not be removed by extraction with acetone.

5. After treating the skin with BAL (2:3-dimercaptopropanol), all the arsenic could be extracted with acetone. Injection of BAL did not produce this effect.

6. BAL did not appear to affect the rate at which the vesicants were absorbed.

Permission from the Director General of Scientific Research (Defence), Ministry of Supply, to publish this work is gratefully acknowledged.

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British Anti-Lewisite as an Antidote for Acute Mercury Poisoning

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In another paper of this series (Thompson & Whittaker, 1947) a review is given of the application of BAL to the treatment of intoxication by metals other than arsenic. Using the brain pyruvate enzyme as a test system these authors showed that *in vitro* BAL was able to protect against the toxic action of compounds of antimony, gold and mercury. Concurrently with this work experiments were carried out to determine whether BAL was also effective *in vivo* as an antidote to injected or orally administered mercuric chloride.

Many authors (Rosenthal, 1933, 1934; Wolpaw & Alpers, 1942; Rozhkov & Arkhipov, 1939; Barnes, 1939; Hug, 1938) have claimed that rongalite (sodium formaldehyde sulphoxylate) is an effective antidote for mercuric chloride poisoning. On the other hand, Modell, Gold, Winthrop & Foot (1937) reviewing the evidence in conjunction with their own experimental results came to the conclusion that 'while sulphoxylate may prove of some clinical value in reducing the mortality in bichloride of mercury poisoning, the problem of controlling the chief cause of the high mortality in human poisoning remains unsolved'. They found that when the mercury and the antidote were given orally to cats no appreciable protection was afforded when the delay in treatment was longer than 15 min. Munoz (1935) working with rats found very little protection after 5 min. In view of this doubtful value of rongalite an attempt was therefore made to assess the relative merits of this substance and BAL in acute oral poisoning.

EXPERIMENTAL AND RESULTS

BAL therapy in mercury poisoning

Acute mercury poisoning was produced in rats by intraperitoneal injection of an aqueous solution of mercuric chloride; half of the animals were used as controls and after an interval of 10 min. the other half were given a solution of BAL in benzyl benzoate-peanut oil mixture as made up for therapeutic use (Eagle, 1943). To avoid local detoxication, the antidote was injected intramuscularly into the hind leg.

It will be seen from Table 1 that the dosage-mortality curve for $HgCl_2$ is very steep and that a high degree of protection is afforded by BAL against an amount of mercuric chloride greater than that required to produce 100% fatalities. These experiments clearly show that BAL is an effective antidote to mercury when introduced by injection into the animal and it was considered desirable to see if a similar degree of protection could be obtained when the poison was given orally. In order to minimize variations in the results it was found necessary to employ the following conditions:

Table 1. *The effect of intramuscular-injected BAL against injected mercuric chloride*

No. rats	Dose $HgCl_2$ (mg./kg.)	Dose BAL (mg./kg.)	Survivors No.	Survivors (%)
10	10	Nil	0	0
10	5·5–5·75	Nil	0	0
7	4	Nil	0	0
10	2	Nil	10	100
10	10	50	1	10
10	5·9–6·25	50	9	90
9	4·0–4·2	50	9	100
10	2	50	10	100

(1) The treated and untreated animals of a particular experiment were selected from rats with the same history.

(2) The rats were starved for 20–24 hr. before commencing the experiment.

(3) A post-mortem examination was carried out on all the animals which died and on those which survived the observation period of not less than one month. The post-mortem examination was necessary to ensure that no complicating factors were present; for example, it was noticed that in a batch of rats received on one occasion a few of the animals had lung abscesses; all such batches were excluded.

The mercuric chloride, usually a 1% solution, was given by stomach tube using a No. 3 gum elastic catheter. For oral administration a fresh aqueous solution of BAL was employed and for injection the standard 5% preparation in oil. Table 2 gives the toxicity figures for mercuric chloride, Table 3 the results with BAL given orally and Table 4 with BAL by intraperitoneal injection.

From these results it appears that absorption of the mercury is too rapid to allow local detoxication in the stomach (Table 3) and that it is essential to introduce the antidote more quickly into the circulation (Table 4). In this case significant protection can be obtained at 15 min., but it was felt that the therapy might be improved by a combination of the

two techniques especially since post-mortem examination revealed local damage in the stomachs of injected animals.

Table 2. *The toxicity of mercuric chloride given by mouth*

No. animals	Dose (mg./kg.)	Survivors No.	Survivors (%)
8	6	8	100
7	12	7	100
8	24	8	100
12	50	4	33
12	75	0	0
40	100	1	2·5
6	150	1	17
31	200	0	0

Table 3. *The effect of BAL against mercuric chloride (both orally administered)*

No. animals	Dose $HgCl_2$ (mg./kg.)	Dose BAL after 15 min. (mg./kg.)	Survivors No.	Survivors (%)
6	50	75	0	0
12	75	75	2	17
6	100	75	0	0
6	150	75	0	0
6	200	75	0	0

Table 4. *The effect of BAL (intraperitoneal injection) against mercuric chloride (given orally)*

No. animals	Dose $HgCl_2$ (mg./kg.)	Dose BAL (mg./kg.)	Time of BAL injection (min.)	Survivors No.	Survivors (%)
6	50	75	15	6	100
12	100	75	15	8	75
12	100	75	30	3	25
12	100	75	60	5	41

Before commencing an extensive series of experiments on the combined therapy, a comparative test was carried out to see which of three injection routes gave the best results.

Three groups of rats were given 100 mg./kg. $HgCl_2$ by mouth and 30 min. later, BAL (50 mg./kg.) was injected either intraperitoneally, intramuscularly or subcutaneously. At 120 min. each of the groups received 25 mg./kg. oral BAL. All animals survived, but at the end of 3 weeks the subcutaneous group showed a loss in weight while the other two groups showed a gain. On the basis of the figures given in Table 5 it seems there is little to choose between intramuscular and intraperitoneal injection, but in view of the slight advantage shown by the latter this method was the one used in subsequent experiments. For technical reasons, intravenous injections were not attempted, but it is possible that the use of BAL-INTRAV may lead to an improved therapy.

Table 5. *A comparison of various injection techniques in the treatment of oral poisoning with mercuric chloride*

No. animals	Dose $HgCl_2$ (mg./kg.)	Injection route	Initial gross wt. (g.)	Gross wt. change (g.)
6	100	Intraperitoneal	1516	+ 48 (3%)
6	100	Intramuscular	2087	+ 12 (0·6%)
6	100	Subcutaneous	1460	− 158 (− 10·8%)

In order to allow some degree of comparison between the various methods of applying BAL as an antidote, the amount of BAL used was kept constant at 75 mg./kg.; thus, when two routes were employed 50 mg./kg. were injected and 25 mg./kg. were given orally. The results obtained with the combined therapy (Table 6) indicate an enhanced survival rate

Table 6. *The effect of BAL (oral and injected) against mercuric chloride (oral)*

No. animals	Dose $HgCl_2$ (mg./kg.)	Time before injection of BAL (min.)	Time before oral dosage with BAL (min.)	Survivors No.	Survivors (%)
12	50	15	30	12	100
12	100	15	30	12	100
9	100	30	120	7	78

as compared with the single injection method. It might be argued that this improvement was merely due to the division of the dose with the result that a concentration of the drug was maintained over a longer period of time; this was shown not to be the case by an experiment in which nine animals were injected at 15 min. with 50 mg./kg. and at 30 min. with 25 mg./kg.; of these animals, only four survived.

The effect of rongalite on oral mercuric chloride poisoning

Although in the past the best results appear to have been obtained by the intravenous injection of rongalite, it was considered worth while to compare

Table 7. *Rongalite in the treatment of mercuric chloride poisoning*

No. animals	Dose $HgCl_2$ (mg./kg.)	Rongalite treatment Dose (mg./kg.)	Rongalite treatment Time before dosage (min.)	Survivors No.	Survivors (%)
12	50	400	15 (oral)	6	50
9	100	400	15 (oral)	0	0
12	50	100 100	15 (intrap.) 30 (oral)	8	66
9	100	100 100	15 (intrap.) 30 (oral)	0	0

rongalite with BAL when administered under the same conditions. The rongalite was made up in bicarbonate solution as recommended by Modell *et al.* (1937) and the results are given in Table 7.

DISCUSSION

The evidence presented in the previous paper by Thompson & Whittaker (1947) demonstrates that BAL is able to protect the pyruvate oxidase system of brain from the toxic action of mercury. Since at the time it was not possible to carry out *in vitro* experiments designed to show whether BAL was also able to bring about a reversal of the inhibition it was decided to expedite the practical application of BAL by direct trial *in vivo*. It has been found that intramuscular injection of BAL is an effective antidote to acute systemic poisoning caused by injection of mercuric chloride. Similar work (quoted by Waters & Stock, 1946) has also been carried out in the U.S.A. Although acute mercury poisoning in man can be produced by contact, it usually occurs by ingestion, and it was considered of interest, therefore, to investigate in rats the effect of BAL and also of rongalite against orally administered mercury. When the compounds were given by mouth, no significant change was observed in the survival rate (Tables 2, 3 and 7). When, however, the antidotes were given by the combined injection and oral technique the difference in the results was striking. Thus, after dosing with 100 mg./kg. $HgCl_2$, 39 of a group of 40 rats died, whilst complete survival was obtained in the BAL group of 12; of nine rongalite-treated rats all died. These figures do not necessarily prove that the BAL has brought about a complete reversal of the toxicity, but taken in conjunction with the evidence of Barron & Kalnitsky (1944), who found that BAL was able to reactivate succinoxidase poisoned with 4-chloromercuribenzoic acid, and of Bloom (1943), who used BAL to prevent the development of vesication caused by 2-thienylmercuric chloride, they do suggest that partial reactivation at least has taken place. From this work it appears that the conclusions reached by Modell *et al.* in respect of the therapeutic value of rongalite are substantiated and that BAL is a more effective antidote for the treatment of acute systemic mercuric chloride poisoning.

SUMMARY

1. Acute mercury poisoning in $HgCl_2$-injected rats is prevented by injection of BAL.

2. Rats are also protected from a lethal dose of $HgCl_2$ given by mouth if BAL is administered by injection and supplemented by oral dosage. This treatment is superior to rongalite given under the same conditions.

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A Note on the Microdetermination of British Anti-Lewisite

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The introduction of 2:3-dimercaptopropanol (BAL) for the treatment of systemic arsenical intoxication in man made it desirable to study the distribution of BAL in the body after injection, and also the rate and mode of its excretion. A method for the microdetermination of BAL was therefore required, and the present note describes an improvement of a procedure suggested by Calvery (1943), and its application to plasma, blood and urine.

EXPERIMENTAL

Microdetermination in simple aqueous solution

In 1943, Calvery reported the successful determination of small amounts of BAL using the colour formed by BAL with cobalt nitrate, but he gave no details of the method, and had applied it only to aqueous solution and to the aqueous humour of the eye.

In a preliminary study of the reaction carried out in this laboratory it was found that the intensity of the colour formed with cobalt nitrate by a given amount of BAL was increased by making the solution more alkaline before addition of the BAL. Warming the solution before adding the BAL had a similar effect. It was also found necessary to add a stabilizing colloid such as gum arabic to prevent the solutions turning cloudy on standing. Thus the pH and the temperature of the solutions before addition of the BAL had to be controlled, and the following final method was therefore adopted.

Cobalt nitrate solution (1 ml. of 0·5 %) was mixed with 0·5 ml. 2 % gum arabic solution and sufficient 0·1 N-borate buffer (pH 9) to bring the final volume, including the BAL solution, to 10 ml. This reagent solution was heated to 45° in a water-bath, the BAL solution was added, and the mixture was allowed to stand at 45° for 10 min. The colour intensity was then measured with a Pulfrich photometer using light of wave-length 4700 A., this being the wave-length of maximal light absorption of the colour.

Linear proportionality between colour intensity and BAL concentration was obtained under these conditions in the range 10–200 μg. of BAL. The solutions were heated only to 45° because a precipitate, which would have interfered with the colour estimations, was formed at higher temperatures.

In the later work with biological fluids, acetic acid was added to stabilize the BAL, and the presence of this acid in aqueous BAL solutions caused a slight fall in colour intensity. This fall was not due to a shift in pH caused by the acid, the buffering capacity of the solution being sufficient to maintain the pH at 9·0. Physiological concentrations of NaCl did not interfere with colour development. The method gave satisfactory recoveries (96–102 %) using aqueous solutions of BAL of known strength.

Specificity of the method. Other thiols give colours with cobalt nitrate under the conditions described, and although in many cases the colours are indistinguishable by the naked eye from the colour given by BAL, the points of maximum light absorption are usually slightly different from that of the BAL colour. The intensities of the colours, calculated on a basis of SH content, vary widely.

Applications of the method to plasma, blood and urine

Added BAL could not be recovered quantitatively from plasma, blood or urine, but in view of the highly reactive nature of 1:2-dithiols, it seems likely that the losses represent actual destruction of part of the BAL, rather than any failure in the method of estimation.

If BAL is added to plasma (containing NaF to prevent clotting), and the mixture is acidified and ultrafiltered, 65–70 % of the BAL can be recovered. The losses are probably due partly to oxidation by air, a process which occurs in simple aqueous solution at physiological pH, and which would be catalyzed by traces of iron porphyrins and metals in the plasma (Barron, 1943). In addition, some BAL apparently reacts with proteins, since only 70 % of added BAL could be recovered from an artificial 7 % protein solution.

To estimate recoveries from blood, BAL was mixed with the blood, the red cells were removed by centrifugation and the plasma acidified and ultrafiltered. This method gave average recoveries of between 30 and 69 %, depending on the initial concentration of BAL. In blood, the main cause of destruction is probably oxidation by haemoglobin within the red cells (Barron, 1943).

If normal urine containing BAL is added directly to the cobalt solution, average recoveries of between 56 and 88 % are obtained. Urine containing albumin yielded recoveries of 60–81 %, the measurements being carried out with the extracts after precipitation of the proteins with trichloroacetic acid. In every case, an increase in the initial concentration of BAL produced higher recoveries. In urine the losses seem to be due to reaction of BAL with some oxidizing agent, an equivalent amount of a reducing substance being liberated, since while the recoveries by the cobalt method are low, practically quantitative recoveries are obtained by iodine titration.

SUMMARY

1. A method is described for the micro determination of BAL, using the colour formed by BAL with cobalt nitrate.

2. The method has been applied to the measurement of the recoveries of added BAL from plasma, blood and urine. Although it has not been found possible to obtain a quantitative recovery from any of these fluids, the losses appear to be due to destruction of BAL by chemical reaction rather than to any defect in the method of estimation.

This work was carried out as part of a programme of extra-mural research for the Ministry of Supply, under the direction of Prof. R. A. Peters, M.C., F.R.S. Thanks are due to the Chief Scientist, Ministry of Supply, for permission to publish, and to Prof. Peters, Dr L. A. Stocken, and Dr R. H. S. Thompson, for their help and advice.

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Further Investigations on the Metabolism of 2:3-Dimercaptopropanol

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Although 2:3-dimercaptopropanol (British Anti-Lewisite, BAL) was first introduced (Stocken & Thompson, 1941, 1946) as a local antidote for use after contamination of the skin with one of the arsenical vesicants, its application to the treatment of systemic arsenical poisoning in man made it desirable to obtain information concerning the distribution of thiols in the blood and urine after injection of BAL.

In this laboratory (Thompson & Stocken, 1941) it had been shown that shortly after the injection of BAL into rabbits and rats the urine of the animals developed a strong nitroprusside reaction for thiol groups, accompanied by a large increase in the iodine titre of the urine. The nitroprusside reaction, which is ordinarily negative, again disappeared and the iodine titre fell to its previous value within a few hours of the injection.

In order to obtain more precise information regarding the nature of the urinary thiols, the cobalt method for the estimation of BAL (Calvery, 1943; Spray, 1947) has been applied to the urine of rabbits after injection of BAL. Owing to the limited specificity of this reaction (Spray, 1947), a series of experiments was carried out in which the reactions of BAL with salts of other metals were examined, and compared with those given by the thiol appearing in the urine after injection of BAL. These experiments, however, did not by themselves provide conclusive evidence as to the nature of the thiols present in rabbit urine after injection of BAL, and further evidence was therefore sought.

The extraction and purification of the thiols from rabbit urine were investigated, and a procedure was evolved, based on the precipitation of the thiols with the thallous ion in alkaline solution, and subsequent decomposition of the metal complex with H_2S. In this way relatively pure and concentrated preparations of urinary thiols could be obtained.

The reactions with metal salts were then re-examined using the purified thiol, and in addition, through the kindness of Mr E. R. Holiday, a comparison of the absorption spectra of the colours formed by reaction of the cobalt reagent with BAL and with the excreted thiol was made, in order to obtain more precise information regarding the identity of the coloured derivatives.

The thiol was then tested for its ability to protect the pyruvate oxidase enzyme system of brain from inhibition by lewisite. By this means it was hoped to obtain biochemical evidence which would help to characterize the excreted thiol as either a mono- or a dithiol, since it has been shown (Stocken & Thompson, 1941, 1946) that this enzyme system can be completely protected from the toxic action of lewisite oxide by dithiols at concentrations at which all the monothiols that have been studied are completely ineffective (Sinclair, 1940).

EXPERIMENTAL AND RESULTS

The estimation of thiols in rabbit urine after injection of BAL

Male rabbits weighing 1·5–3·5 kg. were catheterized, using a no. 3 soft rubber catheter, and the bladder emptied. After 1½–2 hr. the animals were again catheterized, and a sub-lethal dose of BAL dissolved in propylene glycol was injected intramuscularly into one of the hind legs. The bladder contents were drawn off by catheter at suitable intervals after injection.

One part of each urine sample was titrated with 0·02N-iodine after acidification, and another part was either ultra-filtered or treated with trichloroacetic acid to remove any protein. The protein-free extracts were added to the cobalt nitrate reaction mixture, and the colour that developed was measured in a Pulfrich photometer as described by Spray (1947). The urine sample obtained over a known time interval immediately before injection of the BAL was used to compensate for the interfering effect in the colour estimations of the urinary pigments, and of any products of reaction between the normal urinary constituents and the cobalt reagent. A similar allowance was made for the iodine titre of the pre-injection urine in calculating the BAL output from the iodine titration.

Table 1 gives the results obtained on a series of rabbits, the colour-producing thiols being expressed as mg. BAL. It will be seen that whereas the iodine titration gives a recovery of 10–30 % of the injected BAL during the first 5–8 hr. after injection, the cobalt method accounts for less than 10 %. The recovery of thiols by the cobalt method is of the order of 10–20 % of that by iodine titration.

From the above results it is clear that there is present in the urine of rabbits, after injection of BAL, a thiol capable of reacting with cobalt nitrate

Table 1. *Estimation of thiols in rabbit urine after injection of BAL by the cobalt method and by iodine titration*

A. Cobalt method

Rabbit no.	Amount of BAL (mg./kg.)	BAL excreted during the interval stated (mg.) 0–1½ hr.	1½–3½ hr.	3½–5 hr.	Total
1	40	1·6	4·5	0·04	6·1
2	40	0·3	0·9	0·01	2·3
3	40	1·2	2·0	0·01	3·2
4	50	0·3	1·6	0·7	2·6
		0–2 hr.	2–4 hr.	4–8 hr.	
5	50	5·7	0·6	0	6·3

B. Iodine titration

		0–1½ hr.	1½–3½ hr.	3½–5 hr.	5–8 hr.	
1	40	6·4	20·0	2·8	—	29·2
2	40	3·4	7·5	0·7	—	11·7
3	40	5·0	7·5	1·0	—	13·5
4	50	5·5	12·5	6·6	2·3	26·9
		0–2 hr.	2–4 hr.	4–8 hr.		
5	50	18·9	4·3	1·4		24·6

Rabbit no.	Percentage amount injected recovered by: Cobalt method	Iodine titration	Iodine recovery given by cobalt method (%)
1	5	24	21
2	1	8	11
3	4	19	24
4	2	18	10
5	7	29	26

to give a colour very similar to that given by BAL itself. A preliminary measurement of the light absorption at different wave-lengths of the colour given by the post-injection urine with the cobalt reagent suggested, however, that the thiol contained in the urine might not be BAL itself, since the maximum light absorption occurred at a different wave-length from that of the BAL colour. In view of this an attempt was next made to isolate and identify the thiol present in post-injection urine.

Extraction and attempted isolation of thiols from post-injection urine

In the hope of obtaining larger amounts of post-injection urine, specimens were collected from rabbits in metabolism cages. It was found, however, that the urine obtained by this method gave only a faint nitroprusside reaction, due possibly to destruction of the thiols by contact of the urine with the metal of the metabolism cages. Waxing of the metal to prevent this contact, however, failed to improve the yield. We reverted therefore to catheterization, which at the same time obviated the necessity for the urine standing for variable periods of time exposed to air.

In the first attempt to isolate the excreted thiols, the acidified urine was saturated with ammonium sulphate and extracted with benzene. This method did not yield satisfactory results, since it was found that the partition coefficient of the urinary thiol differed from that of pure BAL added to urine. Thus, if post-injection urine is saturated with ammonium sulphate and shaken with 0·25 vol. of benzene, only 20 % of the thiol passes into the benzene layer in the first extraction, instead of 50 % as is found with BAL added to urine.

An attempt was next made to precipitate the thiol with a metal salt. In the course of a qualitative study of mercaptide formation by BAL with metal salts, it was found that while most of the heavy metal mercaptides were insoluble at pH 1, the thallium mercaptide was soluble, precipitation only beginning at pH 5. It was expected therefore that BAL would be regenerated by decomposition of this mercaptide with H_2S.

The method adopted was as follows:

Excess of saturated thallous sulphate solution was added to the acidified urine, followed by N-NaOH drop by drop until precipitation of the yellow mercaptide was complete (pH 7·8–8·0). The solution was then warmed to 50°, with constant stirring, until the precipitate flocculated, after which it was filtered and washed with water. Too intense heating was avoided, since it caused the formation of a compact granular precipitate which was difficult to resuspend in an aqueous phase for decomposition.

The precipitate and filter paper were ground up in a small amount of 0·2N-acetate buffer (pH 5·7), and H_2S was passed until precipitation of the sulphide was complete. The solution was warmed gently, then filtered and the precipitate washed. The filtrate, containing the thiol, was acidified to Congo red, and excess H_2S removed *in vacuo*. In later experiments centrifugation was substituted for filtration at each stage.

Identification of purified thiol in post-injection urine

A comparison was made of the optical densities, at different wave-lengths, of the cobalt colours given by the thallium-purified thiol from post-injection urine and by BAL added to normal rabbit urine, and recovered from it by the thallium procedure. The optical densities are given in Table 2, from which it will be seen that the wave-length of maximum light absorption for the purified excreted thiol differs from that given by BAL.

Table 2. *Optical densities of the cobalt colours given by the purified excreted thiol ('metabolized BAL') and by BAL added to normal rabbit urine and recovered ('recovered BAL')*

Wave-length (A.)	Optical densities ($E_{1\,cm.}$) for 'Metabolized BAL' (*a*)	'Recovered BAL' (*b*)	$E/E_{max.}$ ratio *a*	*b*
4300	0·11	0·20	1·0	0·97
4700	0·09	0·21	0·79	1·0
5000	0·07	0·17	0·59	0·83
5300	0·03	0·10	0·30	0·48
5700	0·02	0·06	0·18	0·27
6100	0·005	0·03	0·05	0·14
6600	0·0	0·0	0·0	0·0
7200	0·0	0·0	0·0	0·0
7500	0·0	0·0	0·0	0·0

The 'metabolized BAL' solution contained thiols equivalent to 100 μg. BAL/ml. as measured by iodine titration. The 'recovered BAL' solution contained 103 μg. BAL/ml.

A solution of the 'metabolized BAL' gave a colour intensity which, for the 4700 A. band, was *c.* 2·5 times less than the intensity produced by a solution of BAL giving the same iodine titre; this fact provides some explanation of the differences found in the early experiments between the recoveries of thiol calculated as BAL from the iodine titration and from the cobalt method. Further, if an aqueous solution of the thallium-purified thiol is saturated with $(NH_4)_2SO_4$ and shaken with 0·25 vol. of benzene, only 50 % of the thiol passes into the benzene layer in the first extraction, as opposed to 90 % with aqueous BAL (Peters, Stocken & Thompson, 1944). All these experiments provide confirmation for the view that the excreted thiol is closely related to BAL, but is not BAL itself.

In view of the limited specificity of the reaction between BAL and cobalt, a survey of the reaction of many thiols (both mono- and 1:2- and other dithiols) with different metal salts was undertaken in the hope of obtaining reactions which would be of use in characterizing thiols of different types. Briefly, no reaction or series of reactions with metal salts was found capable of differentiating between mono- and dithiols, or by which BAL could be conclusively distinguished from other thiols. It was found, however, that the thallium-purified metabolized BAL gave reactions very similar to those given by BAL itself. A summary of the more important reactions is given in Table 3.

Absorption spectra of the colours given by reaction of BAL and the excreted thiol with cobalt nitrate

To confirm the inference drawn from measurements with the Pulfrich photometer that the thiol extracted from post-injection urine was of a slightly different nature from BAL, absorption spectrum measurements on the cobalt colours of the two thiols were carried out by Mr E. R. Holiday.

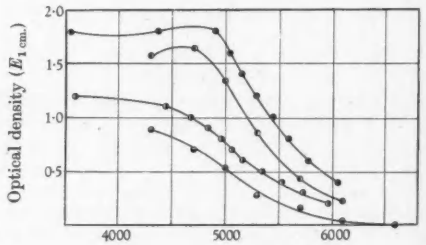

Fig. 1. Chart of the absorption spectra of solutions containing the coloured compounds formed by reaction between BAL and the excreted thiol, and cobalt nitrate. ● absorption spectrum of 'recovered BAL'; ◐ Pulfrich estimation with 'recovered BAL' (1 cm. cell, 800 μg./ml.); ◑ absorption spectrum of 'metabolized BAL'; ◒ Pulfrich estimation with 'metabolized BAL' (1 cm. cell, 800 μg./ml.); all solutions 0·013 N by iodine titration.

Table 3. *Some metal reactions of BAL and 'metabolized BAL'*

Metal	Reactions (at pH 5·7) of (*a*) BAL	(*b*) 'Metabolized BAL'
Fe^{++}	Pink coloration, fading slowly	Pink coloration, fading slowly
Co	Dark brown colour and ppt.	Dark brown colour and ppt.
Ni	Chocolate brown colour	Greenish-brown colour
Hg^{++}	Gelatinous white ppt.	Gelatinous white ppt.
Tl^{+}	Golden yellow ppt.	Yellow ppt., but paler than the BAL ppt.
Cu^{++}	Dark blue colour and ppt., fading to a dirty blue colour	Dark blue colour and ppt., also fading on standing
Pb^{+}	Pale yellow ppt.	Cream ppt.

The thallium method was used to obtain a solution of the excreted thiol present in rabbit urine, and also of BAL when added to normal rabbit urine. The resulting solutions were titrated with iodine and diluted to 0·013N; assuming that only BAL is present this corresponds to 800 μg./ml. Each solution (0·5 ml.) was then added to the usual cobalt reaction mixture, and the visible and ultra-violet absorption spectra measured in the photographic spectroscope.

Fig. 1 shows the variation of intensity of absorption at different wave-lengths calculated for a stratum of 1 cm. thickness. It will be seen that while the colour given by 'recovered BAL' showed a poorly defined maximum in the region of 4700 A. no such maximum occurred in the case of the 'metabolized BAL'. Also, the colour intensity produced by BAL was considerably greater than that produced by an equivalent amount of the urinary thiol.

For purposes of comparison, the optical densities, as measured in the Pulfrich photometer, for solutions of BAL and the excreted thiol at the same strengths as the solutions used for the absorption spectrum measurement, are also included in Fig. 1.

Ability of the excreted thiol to protect the brain pyruvate oxidase system from inhibition by lewisite

As already pointed out, thiols differ markedly in their capacity to protect the brain pyruvate oxidase system from the toxic effects of lewisite oxide, monothiols giving no protection in concentrations at which dithiols protect the system completely. This finding indicates that experiments with this system might provide useful information regarding the nature of the thiol excreted after injection of BAL.

The manometric measurement of the enzyme inhibition in the presence and absence of the protecting thiol was carried out using the usual Warburg technique (Passmore, Peters & Sinclair, 1933; Stocken & Thompson, 1946). In the earlier work it was shown that 10 μg. of lewisite/Warburg bottle (containing 100–150 mg. brain in 3 ml. Ringer phosphate-pyruvate solution) gave approximately 50 % inhibition of enzymic activity; the same concentration of lewisite was used in this work.

In the first experiment 0·3 ml. of untreated post-injection urine was added to one pair of bottles to discover whether any protection of the enzyme system was brought about. As controls, further pairs of bottles were set up, containing respectively, 0·3 ml. of pre-injection urine, and the same quantities of pre- and post-injection urine in the absence of lewisite, in order to allow for any possible toxic effect of the urine itself. The post-injection urine (0·3 ml.) had an iodine titre equivalent to 800 μg. of BAL in excess of the pre-injection specimen. This would be equivalent to 80–200 μg. of BAL as determined by the cobalt method.

In the next two experiments, the thiols were extracted from post-injection urine by thallium precipitation, and the regenerated thiol was added to the bottles. BAL, added to normal rabbit urine and recovered by the thallium method, was used as a control in these experiments. Before addition to the bottles, both the 'metabolized BAL' and the 'recovered BAL' were suitably diluted with Ringer phosphate, so that 1 ml. of the final solution (the amount added to the bottles) contained 100 μg. of BAL, calculated from the iodine titre.

The results of these experiments are given in Table 4. It will be seen that both 0·3 ml. of the post-injection urine, and 100 μg. of the extracted urinary thiol (expressed as BAL) almost completely protected the brain pyruvate oxidase system from inhibition by 10 μg. of lewisite. Since no monothiol yet studied has been found to cause any significant protection of this enzyme system at the concentration used here, it may be inferred from these experiments that the greater part of the excreted thiols, extractable with thallium, is dithiol in nature.

The slight toxicity of the thiols in the later stages of the experiments has previously been observed to occur with these amounts of BAL.

Table 4. *Effect of 'metabolized BAL' in protecting the brain pyruvate oxidase system from inhibition by lewisite*

Exp.	Additions	Percentage inhibition of enzyme activity over 0–30 min.	30–60 min.	60–90 min.
893	10 μg. lewisite	45	41	—
	0·3 ml. pre-injection urine	17	18	—
	Lewisite + pre-injection urine	45	37	—
	0·3 ml. post-injection urine	0	26	—
	Lewisite + post-injection urine	0	30	—
894	10 μg. lewisite	52	61	55
	100 μg. 'metabolized BAL'	6	14	9
	100 μg. 'recovered BAL'	0	16	16
	Lewisite + 'metabolized BAL'	0	0	0
	Lewisite + 'recovered BAL'	0	3	11
895	10 μg. lewisite	65	69	63
	100 μg. 'metabolized BAL'	0	21	25
	100 μg. 'recovered BAL'	0	20	21
	Lewisite + 'metabolized BAL'	11	6	0
	Lewisite + 'recovered BAL'	7	9	16

DISCUSSION

From the evidence presented above it appears that, after injection of sub-lethal doses of BAL, rabbits excrete in the urine a considerable quantity of thiol or thiols, representing some 20 % of the thiol content of the injected BAL, and that a considerable proportion of this excreted thiol is present as a dithiol closely related to BAL. The evidence suggests that the dithiol is not BAL itself, and the possibility immediately arises that the body may detoxicate BAL either by one of the already familiar detoxication mechanisms or perhaps by some means not so far encountered in the study of detoxication mechanisms.

SUMMARY

1. The thiols present in rabbit urine after injection of 2:3-dimercaptopropanol (BAL) have been estimated by iodine titration and by reaction with cobalt nitrate.

2. Extraction by means of precipitation with thallous sulphate has yielded a purified preparation of a thiol present in post-injection urine.

3. Examination of the optical densities at different wave-lengths of the cobalt colour given by the purified urinary thiol together with measurements of its absorption spectrum both in the visible and ultra-violet regions of the spectrum suggest that this thiol is closely related to BAL but not identical with it.

4. The urinary thiol is capable of protecting brain pyruvate oxidase preparations from inhibition by lewisite at concentrations at which no monothiol has yet been found to be effective.

This work was carried out for the Ministry of Supply as part of a programme of research under the direction of Prof. R. A. Peters, M.C., F.R.S. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, to Dr V. P. Whittaker for providing us with some of the thiols used in investigating the reactions with heavy metal salts, to Mr E. R. Holiday for carrying out the ultra-violet absorption measurements, and to Miss M. R. Kempson for skilled assistance.

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The Effect of Injected British Anti-Lewisite on Urinary Sulphur and Glucuronic Acid Fractions

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In an earlier paper of this series (Spray, Stocken & Thompson, 1947) it was shown that the injection of 2:3-dimercaptopropanol (BAL) into rabbits causes a marked increase in the iodine titre of the urine, the latter also developing a strong nitroprusside reaction for thiol groups. The increase in the iodine titre of the urine could have accounted for some 20 % of the injected BAL, and although the excreted thiol was not isolated, strong indications were obtained that it was not BAL but a closely related dithiol. Aqueous solutions of dithiols are rapidly oxidized by atmospheric oxygen at the pH of urine and in view of this it was possible that much more than 20 % was excreted in the urine, a proportion of it being in some oxidized form which would not be measured by iodine titration.

The effects of injection of BAL on the urinary excretion of inorganic sulphate, ethereal sulphate and neutral sulphur were therefore studied in rats, to discover how much of the BAL-sulphur was excreted as neutral sulphur, and also to see whether any was oxidized to inorganic sulphate. In addition, if any BAL were detoxicated in the animal body by conjugation with sulphuric acid, as happens with

many other hydroxyl compounds, there would be an increase in ethereal sulphate excretion after injection.

As it is known that compounds containing hydroxyl groups can also be detoxicated by conjugation with glucuronic acid the urinary excretion of glucuronides has also been followed after administration of BAL.

EXPERIMENTAL

Urinary sulphur partition

Groups of six male rats weighing 200–400 g. each were used. The animals were placed in metal metabolism cages, the funnels of which were coated with paraffin wax to minimize contact of the urine with metal. The rats were transferred to other cages for 2 hr. each day for feeding, urine being collected for the remaining 22 hr. This external feeding eliminated the possibility of contamination of the urine with food, and ensured that a constant amount of food was eaten every day. A 'synthetic' diet of constant composition was used throughout.

Each morning, the funnels of the metabolism cages over which the urine passed were washed with distilled water, the washings being added to the urine. Urine and washings were made up to the smallest convenient volume, filtered clear, and divided into six equal portions for duplicate estimations of the three sulphur fractions.

After several daily determinations of the normal excretions of sulphur fractions had been made, the animals were injected with BAL and the determinations were continued for at least 10 days after injection. In order to vary the injection route, the rats were divided into four groups, groups 1 and 2 receiving 100 mg. BAL/kg. body wt., intramuscularly into a hind leg, and groups 3 and 4 receiving 80 mg./kg. body wt., subcutaneously into the flanks. The BAL was freshly dissolved in 0·9% saline, and the strengths of the solutions were estimated by iodine titration immediately before use.

Inorganic and total sulphate were estimated by the methods of Folin (1905) and total sulphur by the method of Benedict (1909), as modified by Givens (1917). The latter method gave theoretical yields of barium sulphate with known amounts of pure BAL.

The results obtained with the first two groups are shown in Figs. 1 and 2. It will be seen that the injections caused a big rise in neutral sulphur excretion on the day following injection, after which it returned to normal. The increases in neutral sulphur over the means of the normal values represented some 60% of the sulphur injected as BAL. The injections had no significant effect on ethereal sulphate excretion, but there was a marked rise in inorganic sulphate excretion occurring on the day following injection and persisting for at least 10 days. The cumulative increases in inorganic sulphate over the pre-injection levels were far greater than could be accounted for by oxidation of the remaining 40% of the BAL to inorganic sulphate and excretion as such. Thus, in group 1, a total of 77·5 mg. of sulphur was injected as BAL, and in group 2, 72·8 mg. Yet the cumulative excess excretions of inorganic sulphate, apart from the neutral sulphur increase, accounted for *c.* 220 mg. and 270 mg. sulphur respectively. This extra sulphur, which must have arisen from some source other than BAL, could not have come from the diet, since this was constant before and after the injection.

Cuthbertson (1930, 1931), and Cuthbertson, McGirr & Robertson (1939) have found increased excretions of N, S and P following injury in both rats and human beings. Cuthbertson (1931) showed that the increased sulphur excretion was due mainly to an increase in the inorganic sulphate fraction of the urine. It seemed possible, therefore, that the increased inorganic sulphate excretions observed in these experiments were due to increased tissue catabolism caused by injury arising directly or indirectly from the injection. Calvery (1944) has shown that injection of BAL can cause considerable muscle necrosis and other damage, both gross and microscopic.

With this possibility in view, BAL was injected subcutaneously into two further groups of rats, as it was thought that this injection route might cause less tissue damage than that following intramuscular injection. The results of these experiments are given in Figs. 3 and 4. The general course of the excretion rates was similar to that with groups 1 and 2, the rise in neutral sulphur accounting this time for *c.* 40% of the injected BAL. Again there was a rise in inorganic sulphate, not as marked as previously, but still too great to be due simply to day-to-day physiological variation. In these experiments estimations of urinary nitrogen were carried out, and a slight rise in nitrogen excretion after injection was detected, confirming the view that the enhanced excretion of inorganic sulphate is probably largely due to tissue damage caused by the injection.

Glucuronic acid excretion

In preliminary qualitative experiments, in which the urine of rats and rabbits was tested for glucuronic acid by Tollens's (1908) reaction before and after injection of BAL, some evidence was obtained of increased glucuronide excretion after the injection. A quantitative study of the glucuronic acid excretion of animals before and after injection of BAL was therefore undertaken.

For the experiments with rats, groups of three (or in one case, six) large male rats (200–400 g. each) were placed in metal metabolism cages over glass funnels, and were fed outside the cages. The feeding technique, and the treatment of the urine, were the same as already described. The total sulphate, total sulphur and glucuronic acid contents of the urines were determined each day. After two determinations of the normal levels had been made, the animals were given 80 mg. BAL/kg. body wt. dissolved in 0·9% saline, by various routes. Measurements were continued for some days after injection.

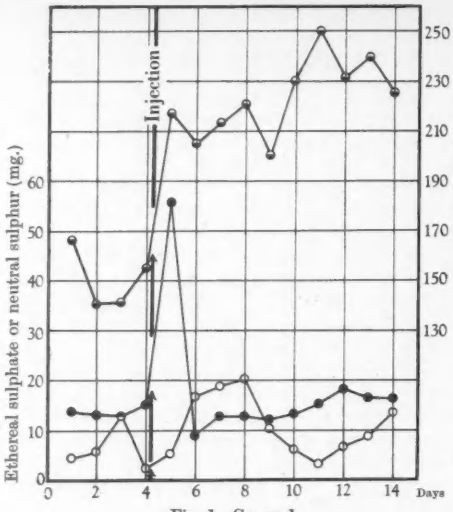

Fig. 1. Group 1.

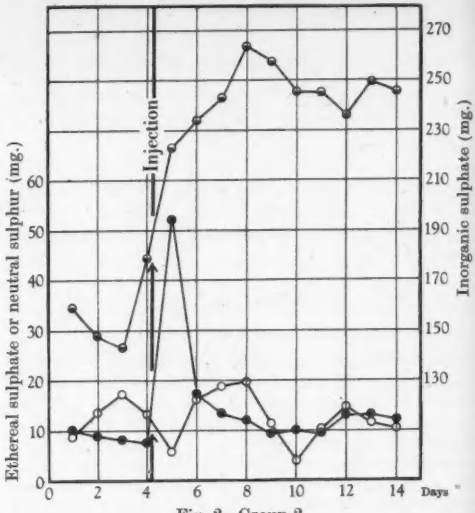

Fig. 2. Group 2.

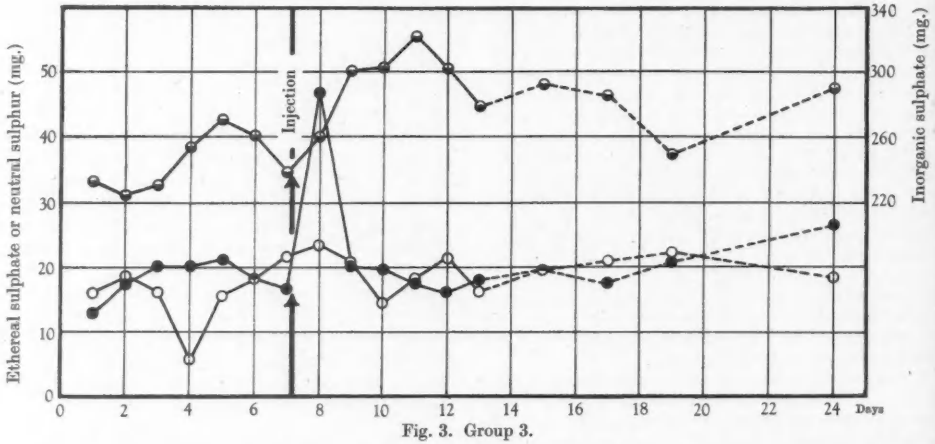

Fig. 3. Group 3.

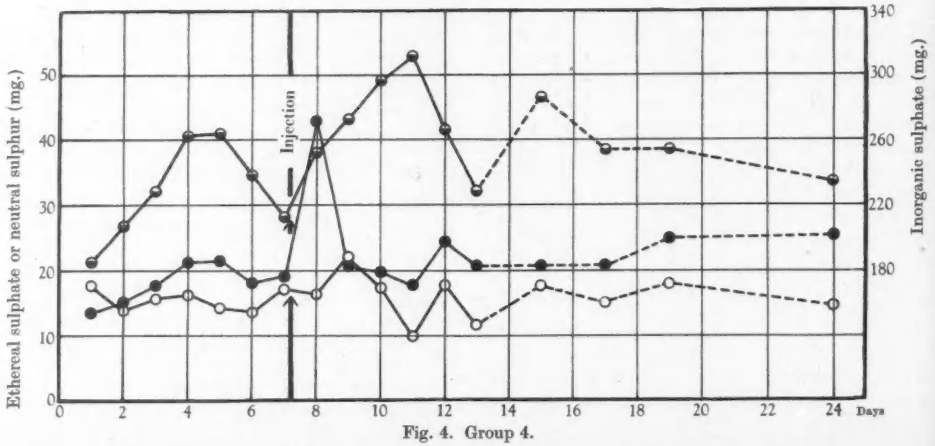

Fig. 4. Group 4.

Figs. 1–4. Urinary sulphate and neutral sulphur excretions of rats before and after injections of BAL.
◒ Inorganic sulphate (as $SO_4^=$); ○ ethereal sulphate (as $SO_4^=$); ● neutral sulphur (as S).

A few experiments with rabbits were also made. Large male rabbits were catheterized and their bladders emptied. They were then given an intramuscular injection of BAL and were catheterized at intervals afterwards. The increases in iodine titre and in glucuronic acid content of the urine after injection were measured.

The estimation of glucuronic acid. Maughan, Evelyn & Browne (1938) and Mozolowski (1940) estimated glucuronic acid by measuring the intensity of the colour developed on heating glucuronic acid with naphthoresorcinol in aqueous solution in the presence of HCl. A modification of this method was used here.

The quantities of reagents and the heating time were the same as those used by the earlier workers. A detailed study of the effects of HCl concentration, naphthoresorcinol concentration, and heating time on the colour intensity confirmed the earlier findings that these quantities and heating time gave the best results. After cooling, the reaction mixtures were diluted with equal volumes of water and were extracted first with 5 ml. and then with 2 ml. ethyl acetate, the ethyl acetate layers being removed by capillary pipettes to graduated 10 ml. cylinders. The combined ethyl acetate extracts were made up to 6 ml. with pure ethyl acetate, and dried with anhydrous Na_2SO_4. The colour intensity was measured in a Pulfrich photometer using a 1 cm. cell and the 5700 A. filter, against a blank obtained by heating and extracting a tube containing water, naphthoresorcinol, and HCl only. The amount of glucuronic acid in the original solution was read off from a calibration curve obtained with known amounts of pure borneol glucuronic acid.

The earlier workers used ether for extracting the colour, and they did not employ the double extraction technique or dry their extracts. It was found that the method used here gave clearer extracts and more complete extraction than could be obtained in any other way.

The method gave a linear relationship between colour intensity and glucuronic acid concentration in the range 10–60 μg. glucuronic acid, and the results were reproducible at intervals of several months. Other constituents of urine did not affect the method at the dilutions necessary to bring the glucuronic acid content within the range of accuracy.

Table 1 shows that in rats there is a marked rise in the urinary glucuronic acid output, accompanied again by an increase in neutral sulphur excretion, during the 24 hr. following the injection of BAL, after which both these excretions return almost to the normal level. The rise in glucuronic acid, relative to neutral sulphur, is much less following intraperitoneal than intramuscular or subcutaneous injection. If the excess excretions of glucuronic acid and sulphur (reckoned as BAL) during the 24 hr. following injection be calculated, it is found that there may be either more or less glucuronic acid excreted than would appear to be required for complete detoxication of the BAL (see molar ratios, Table 1).

In the experiments with rabbits, on the other hand, it was found that the increases in iodine titre, reckoned as BAL, were far in excess of the increases in glucuronic acid in the post-injection urine than in the pre-injection sample. There was also a large excess of thiol over glucuronic acid in the extract obtained by thallium precipitation (Spray, Stocken & Thompson, 1947) of the post-injection urine.

Table 1. *The daily neutral sulphur* (S) *and glucuronic acid* (G.A.) *excretions (in mg.) of rats before and after BAL injections*

Group no.	Injection route	Excretions of	Days before injection: 2	Days before injection: 1	Injection day	Days after injection: 1	Days after injection: 2	Days after injection: 3	Days after injection: 4
1	Subcutaneous	S	21·5	18·1	42·9	20·6	19·9	—	—
		G.A.	68·6	70·7	155·7	73·9	78·6	—	—
2	Intraperitoneal	S	11·6	8·5	28·6	10·9	7·9	7·2	6·4
		G.A.	48·7	42·8	71·4	64·2	50·0	48·7	44·0
3*	Intraperitoneal	S	7·6 (5·1)	7·3 (4·9)	23·7	6·3	6·0	7·3	6·4
		G.A.	35·7 (23·8)	33·9 (22·6)	35·7	27·1	30·4	30·4	25·0
4	Intramuscular	S	6·9	6·9	20·0	4·5	6·6	7·2	—
		G.A.	31·3	32·4	123·0	24·6	36·8	32·4	—
5	Intramuscular	S	10·5	10·9	24·6	7·3	6·9	8·0	—
		G.A.	59·5	60·0	201·8	64·4	53·5	53·5	—
6	Subcutaneous	S	7·3	8·9	24·6	9·8	6·0	6·9	—
		G.A.	49·2	53·4	152·1	49·1	51·4	51·4	—
Group no.			1	2	3	4	5	6	
Molar ratio of excess G.A./excess S (as BAL) on injection day			1·2	0·4	0·2	2·2	3·0	2·0	

* One rat in this group died from the injection. To make some allowance for this, the pre-injection figures have been multiplied by two-thirds and are included in parentheses. The post-injection values refer to two rats only.

The above results indicate that the injection of BAL into rats produces an increased excretion of glucuronic acid in the urine; with rabbits, on the other hand, no such increase can be shown. This provides support for the view that the rat excretes BAL at least partially as a glucuronide, though in the absence of a constant ratio between the excess excretions of BAL and glucuronic acid, it is impossible to say definitely that the excreted compound is a BAL glucuronide.

In view of the large increases in glucuronic acid excretion in rats, the absence of any increased excretion in the rabbit following the injection of BAL is not easy to understand.

SUMMARY

1. Following the injection of BAL into rats, a sharp rise in the urinary excretion of neutral sulphur occurs in the first 24 hr. after injection, the increases representing 40–60% of the sulphur injected as BAL.

2. There is no increase in ethereal sulphate excretion after injection, so that rats do not conjugate BAL with sulphuric acid. However, a marked and persistent rise in inorganic sulphate excretion occurs. This appears to be due rather to increased breakdown of tissues caused by the injury produced by the injection of BAL, than to oxidation of BAL-sulphur to sulphate.

3. Injections of BAL cause increased excretions of glucuronic acid in the urine of rats in the first 24 hr. after injection, suggesting that BAL is excreted as a glucuronide by this species. No such increase was found in the rabbit.

I should like to express my thanks to Prof. R. A. Peters, M.C., F.R.S., Dr R. H. S. Thompson and Dr L. A. Stocken for their help and advice in connexion with this work.

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The Use of British Anti-Lewisite Containing Radioactive Sulphur for Metabolism Investigations

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Various methods have been used for investigating the metabolism of BAL. The nitroprusside reaction and iodine titration (Stocken & Thompson, 1941, 1946) showed that following the injection of BAL there was a rapid increase in the urinary excretion of thiol compounds. An examination of the coloured metallic derivatives suggested the presence of a dithiol, a view which was confirmed by enzyme protection experiments. On the other hand, the ultraviolet and visible absorption spectra of the excreted dithiol showed that this was not unmodified BAL (Spray, Stocken & Thompson, 1947). In the rat, but not in the rabbit, Spray (1947) obtained evidence indicating the possibility of conjugation of the urinary thiol with glucuronic acid but not with sulphuric acid. At the same time, he found an increased excretion of inorganic sulphate which was presumed to be due to increased tissue catabolism caused by local damage at the site of injection. In an attempt to establish this last point with more certainty and to learn more about the distribution, BAL containing S^{35} was tried. Further, since no satisfactory method has been found for the estimation of BAL in blood, it was considered that the 'tracer' technique could provide some information as to the rate of absorption, persistence and amount

in the circulation. The results we obtained were in general agreement with those of the earlier experiments and also with those found independently by Simpson & Young (1945), some of which appeared during the course of our work.

METHODS

The preparation of radioactive BAL

Reduction of $BaSO_4$. An intimate mixture of 125 mg. radioactive $BaSO_4$, 125 mg. $BaSO_4$ and 0·5 g. sugar charcoal was heated at 1000° for 1·25 hr. in a quartz tube. The tube was fitted with a ground-joint tap which was left open during the reduction. After cooling, the BaS was decomposed with the calculated quantity of N-HCl and the H_2S blown by a steady stream of nitrogen into a cooled (−20°) solution of 150 mg. sodium ethoxide in 5·0 ml. ethanol to give a solution containing radioactive sodium hydrosulphide.

Conversion of the radioactive NaSH *into BAL.* Ammonium hydrosulphide was prepared by leading dry H_2S and dry ammonia into anhydrous diethyl ether at −10°. The white crystals were quickly filtered off, pressed as free as possible from ether, and then dissolved in cold methanol. A volume of this solution (130 ml.), containing the equivalent of 8·06 g. H_2S, was mixed with the solution of radioactive NaSH and heated with a 2:3-dibromopropanol (10·9 g.) in a sealed glass bottle for 15 hr. at 90–95°. Excess H_2S and methanol were removed *in vacuo*, the residue made acid to Congo red with 0·1N-HCl and the solution saturated with ammonium sulphate. The product was exhaustively extracted with pure benzene and the extract dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue distilled at low pressure. Yield 3·5 g. (56%) (cf. Stocken, 1947). 'SH' content by iodine titration, 53·1%. Calc. for $C_3H_8OS_2$: 53·2%.

Measurement of the radioactive sulphur. In view of the low energy of the β particles from S^{35} ($1{\cdot}07 \times 10^5$ eV.) all the radioactivity measurements were made with a demountable counter of the type described by Collie & Morgan (1939) in which the radioactive preparation is held inside a movable glass cylinder which can be slid away from the counter for the purpose of determining the background count. After introducing the specimen, the counter was evacuated and filled with a mixture of dry air (at 40 mm. Hg) and ethanol (at 10 mm. Hg). A satisfactory counter can be opened and filled indefinitely without changing its characteristics.

The overall efficiency of the counter was obtained by measuring the activity of the uranium *X* from a known amount of uranium nitrate precipitated on a ferric hydroxide carrier. It was found that 1/14th of the β particles released from the source were registered. It was shown by sliding the source up and down the counter that the sensitive area was sufficiently large for it to be unnecessary to make any special arrangements for locating the source exactly in order to obtain reproducible results.

For measurement, the material was evenly distributed over the inner surface of the cylinder by taking it up in a little water and rotating the cylinder on a horizontal axis while a stream of air was blown through it.

The $BaSO_4$ prepared from radioactive BAL and diluted with four times its weight of $BaSO_4$ was used as a standard. Its activity was repeatedly measured during the course of the work, but after allowing for the natural decay, no significant change in the count was observed.

No correction for absorption in the film was applied; since the thickness of the $BaSO_4$ film was not more than 0·2 mg./cm.² and the penetrating power of the rays in aluminium is several mg./cm.², the omission of this correction is justified.

EXPERIMENTAL AND RESULTS

Investigation of the radioactivity of urine sulphur fractions after BAL injection

Three groups of three male rats were used for the investigation of the urinary sulphur fractions. For intraperitoneal injections, the rats weighed 322, 332 and 265 g.; for intramuscular injections, 284, 289 and 247 g.; and for subcutaneous injections, 243, 281 and 261 g. Each rat received the equivalent of 63 mg./kg. body wt. of radioactive BAL (8% w/v in propylene glycol). The rats were fed outside the metabolism cages for 2·5 hr. (at 10.00–12.00 hr. and 17.00–17.30 hr.) each day. Treatment of the urine for the conversion of the three sulphur fractions to sulphate was carried out as described by Spray (1947).

The results given in Table 1 indicate that the mode of injection has little effect on the S^{35} content

Table 1. *The excretion of* S^{35} *in the sulphur fractions of urine by rats after injection of radioactive BAL*

		Percentage of radioactivity of injected BAL in samples		
Day	Fraction	Exp. 1 (Intraperitoneal injection)	Exp. 2 (Subcutaneous injection)	Exp. 3 (Intramuscular injection)
1	Inorganic SO_4	3·6 (±0·1)*	5·7 (±0·13)	5·25 (±0·1)
	Ethereal SO_4	0·0	0·0	0·75 (±0·02)
	Neutral S	41·6 (±1·9)	40·9 (±1·5)	40·2 (±1·9)
2	Inorganic SO_4	0·4 (±0·05)	0·5 (±0·08)	0·5 (±0·04)
	Ethereal SO_4	0·0	0·1 (±0·02)	0·0
	Neutral S	1·5 (±0·2)	2·0 (±0·2)	1·5 (±0·13)
3	Inorganic SO_4	—	—	0·3 (±0·03)
	Ethereal SO_4	—	—	0·0
	Neutral S	0·4 (±0·025) (total S)	0·9 (±0·07) (total S)	0·9 (±0·1)
4	Total S	0·25 (±0·025)	0·3 (±0·06)	0·3 (±0·025)
5	Total S	—	0·2 (±0·06)	0·3 (±0·04)

* Figures in brackets are the probable error.

of the various fractions. During the first 22 hr. after injection (i.e. day 1) there was a large excretion of S^{35} in the neutral sulphur fraction. Some radioactivity, however, appeared in the inorganic and total sulphate fractions. This might have been due either to direct oxidation of some BAL sulphur to inorganic sulphate, or to exchange of S^{35} between BAL and some thiol compound which was subsequently oxidized to the inorganic sulphate. It is not yet possible to decide between these alternatives. The S^{35} content of the ethereal sulphate fractions was either zero or a very small fraction of the total.

Small amounts of S^{35} continued to be excreted for at least 5 days, indicating that there must be considerable retention of S^{35}, either as BAL or because of exchange, in the body. It was not thought that determinations could usefully be carried on any longer than this, since it was not possible to measure the feeble radioactivity of the late samples with any accuracy. It was for this reason also that no radioactivity measurements were carried out on the inorganic and total sulphate fractions after the second or third day.

Distribution of S^{35} after injection of radioactive BAL

A large male rat (320 g.) after feeding on a synthetic diet for 7 days was starved for 24 hr. and then injected intramuscularly in the hind leg with 32 mg. radioactive BAL dissolved in 0·4 ml. propylene glycol. 1 hr. later it was killed by decapitation, and sufficient blood collected for estimation of the S^{35} content. Other organs and parts of the rat were dissected out. In this dissection great care was taken to avoid contamination of S^{35} between the various tissues. The skin was freed from subcutaneous fatty and connective tissues by scraping. Muscle was taken from the hind leg not used for injection.

The weighed tissues were placed in Kjeldahl flasks, covered with 10–20 ml. conc. nitric acid and allowed to stand overnight. This caused the tissues to disintegrate, so avoiding the risk of explosion which sometimes occurs if heating is begun directly. To the mixture were added 5–10 ml. perchloric acid (60%) and the digestion carried out in the usual way, with the addition of a few drops of nitric acid from time to time. The sulphuric acid was precipitated as $BaSO_4$, which was collected, weighed and a convenient amount taken for the count. Almost theoretical yields of $BaSO_4$ were obtained by this method in a control experiment in which a known amount of BAL was oxidized in the presence of liver.

From Table 2 it can be seen that the kidney, liver and small intestine had a greater concentration of BAL calculated from the S^{35} content than the other organs. This distribution is probably due to rapid elimination in the urine and the bile.

Table 2. *The distribution of S^{35} and BAL in the body of a rat after intramuscular injection*

	Wt. of tissue (g.)	S^{35} content (% of amount injected)	BAL (μg./g. tissue)
Eyes	0·33	0·02 (±0·002)*	19·4
Brain	1·74	0·17 (±0·002)	31·3
Stomach and contents	1·83	0·17 (±0·002)	29·7
Small intestine and contents	7·17	3·6 (±0·05)	161
Large intestine and contents	3·89	0·68 (±0·007)	56·0
Heart	1·21	0·13 (±0·003)	34·4
Lungs	1·39	0·21 (±0·002)	48·4
Liver	9·08	2·8 (±0·04)	98·6
Kidneys	2·46	2·1 (±0·04)	274·0
Spleen	1·13	0·20 (±0·002)	56·6
Testes	1·31	0·15 (±0·003)	36·6
Skin	40·1	0·46 (±0·01)	3·66
Muscle (remote)	3·07	0·30 (±0·004)	31·2
Injection leg	29·8	20·1 (±0·35)	21·6
Blood	4·77	—	55·0

* Figures in brackets are the probable error.

Persistence of S^{35} in the blood after injection of radioactive BAL

A large female rabbit (3100 g.) was given 160 mg. radioactive BAL dissolved in propylene glycol by intramuscular injection into the hind leg. At hourly intervals, blood was collected from the ear vein, and the S^{35} content estimated.

Table 3. *The distribution of S^{35} and BAL in rabbit blood after intramuscular injection*

Time after injection (hr.)	% of injected S^{35} in total blood wt.*	BAL (μg./g. of blood)
1	5·4 (±0·07)†	33·0
2	5·0 (±0·12)	30·8
3	2·6 (±0·05)	16·1
4	2·5 (±0·04)	15·6
5	2·1 (±0·04)	13·0
6	1·8 (±0·07)	11·2
7	1·7 (±0·04)	10·5

* Total wt. of blood is assumed to be 1/12th of body wt.
† Figures in brackets are the probable error.

From Table 3 it will be seen that the BAL content of blood reached a fairly high level during the first

2 hr. after injection, *c.* 5% of the injected S^{35} being in the circulation; during the next hour it fell to about half this figure and then steadily declined over the next 5 hr.

In both this and the previous experiment, it must be remembered that the BAL content has been calculated from the amount of S^{35} found. The figures, therefore, necessarily give an upper limit to the BAL concentration, since there is the possibility of exchange and irreversible oxidation to non-thiol compounds.

DISCUSSION

There is general agreement in the distribution of S^{35} between the amounts obtained by Simpson & Young (1945) 6 hr. after injection of radioactive BAL and those found by us. With the exception of the small intestine where the percentages of S^{35} were approximately the same (3·7 and 3·61) the concentrations in the other tissues after 6 hr. were, as would be expected, consistently lower. In our experiments there is also fair agreement between the concentrations found in the blood of the rat and rabbit 1 hr. after injection; thus the rat which received twice the dose given to the rabbit had nearly twice the concentration of BAL in the blood.

From the therapeutic point of view it is desirable to determine the rate of absorption of BAL, the level of BAL in the blood stream and for how long an adequate concentration is maintained. The figures given in Table 2 show that 80% of the injected BAL had already entered the circulation within 1 hr. of injection. At this time the maximal concentration had been reached and was maintained for not less than 2 hr. (Table 3); assuming, therefore, that all the S^{35} is due to BAL itself, the data indicate that these doses repeated at intervals of 3 hr. are sufficient to ensure maximum therapeutic effect in rats. This frequency of dosage is consistent with the American recommendations embodying initial dosing of BAL at intervals of 4 hr. for patients suffering from arsenical dermatitis, a treatment which was based upon studies in rabbits (Eagle, 1943) and injection experiments in human volunteers (Sulzberger, Baer, Kanoff & Eagle, 1944).

A further point of therapeutic importance is the confirmation that a single dose of BAL is largely excreted within 6–24 hr. after injection; this means that any possible toxic effects of a single dose should be temporary.

SUMMARY

1. A method is given for the preparation of radioactive 2:3-dimercaptopropanol (BAL).

2. It has been shown that the S^{35} of radioactive BAL is largely excreted in the neutral sulphur fraction of post-injection urine.

3. The distribution and persistence in the circulation of S^{35} following injection of radioactive BAL has been determined.

The radioactive sulphur used in this work was obtained in the form of $BaSO_4$ through the good offices of the Chemical Defence Research Department of the Ministry of Supply from Dr F. C. Henriques of Harvard University, U.S.A., to whom our thanks are due.

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Oxidation of Phenol and Benzoic Acid by Some Soil Bacteria

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The utilization of aromatic compounds by certain bacteria is now well known, and numerous workers have described the isolation of micro-organisms from various sources, capable of metabolizing phenolic substances. Thus, Sen Gupta (1921) has studied quantitatively the disappearance of phenols and cresols from soils of diverse origin, and showed clearly the biological nature of the process. Störmer (1908) had tested the effect on soil of a number of organic compounds, including toluene, xylene, phenol and *p*-cresol, and claimed to have isolated organisms that could destroy these compounds, but

details of this part of the work are not given. Fowler, Ardern & Lockett (1911) suspected the occurrence of phenol-utilizing bacteria on sewage filter beds, because phenol produced in the course of the decomposition of sewage proteins did not accumulate. They isolated from sewage effluent an organism resembling *B. helvolus* (Zimmermann) (*Flavobacterium helvolum*) and found that it grew in a mineral salt medium to which phenol (0·01 g./100 ml. of medium) was added, and destroyed the phenol.

In recent years, the wide use of aromatic compounds as soil insecticides, has stimulated considerable interest in the mechanism of their disappearance. Thus, the Rothamsted workers, Gray & Thornton (1928) and Tattersfield (1928), isolated many types of soil bacteria capable of destroying the following aromatic compounds: phenol, *o*-, *m*- and *p*-cresol, naphthalene, phloroglucinol, resorcinol and toluene, which are commonly used as soil sterilizing agents. Pure cultures of these organisms were capable of utilizing these compounds as sole source of organic carbon, when incorporated into a mineral salt medium, containing an inorganic nitrogen supply. In a study of the distribution of these micro-organisms, the above authors showed that they were most often found in arable soil, but rarely occurred in unmanured or 'wild' soil. From a study of their morphology and growth characteristics, the strains were classified into the following six families: *Coccaceae*, *Mycobacteriaciae*, *Bacteriaceae*, *Pseudomonadaceae*, *Spirillaceae* and *Bacillaceae*, respectively.

In a study of the bacterial purification of gas-works' liquor, Happold & Key (1932) isolated from sewage a Gram-negative vibrio (vibrio 01) which was capable of attacking monohydroxyphenols. The present author has isolated phenol-utilizing organisms from the faeces of a large variety of animals, viz. man, horse, cow, sheep, and pig, and they appear to be normal inhabitants of the intestine.

Little is known of the mechanism and of the intermediate products through which these micro-organisms metabolize benzene derivatives. Happold (1930) has studied the capacity of bacterial suspensions to catalyze the oxidation of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine. He found that the oxidation of catechol with the formation of *o*-benzoquinone, ran parallel with the ability of the organism to give the dimethyl-*p*-phenylenediamine oxidase reaction, as applied by Gordon & McLeod (1928). *Vibrio tyrosinatica* (Beijerinck) was the only organism which possessed tyrosinase, and which consequently catalyzed the oxidation of monohydroxyphenols, with subsequent formation of more complex components. None of the phenol-utilizing organisms isolated by Gray & Thornton (1928), Happold & Key (1932), or the present author, contain a demonstrable tyrosinase system, if the criterion employed be the capacity to oxidize tyrosine, giving the well-known colour sequence, leading to melanin formation, characteristic of the tyrosine-tyrosinase reaction. Tyrosine is however utilized by most of these organisms, but the products formed are quite colourless, and eventually cleavage of the benzene ring occurs.

It would appear, therefore, that the destruction of monohydroxyphenols by these organisms is quite different from the well recognized type of biological oxidation produced by aerobic oxidases, elucidated by Raper and his collaborators (see Raper, 1928).

The present communication is concerned with the further study of the metabolic activities of these micro-organisms, using phenol and benzoic acid as substrates, in an attempt to reveal the mechanism of the oxidative destruction of the benzene ring.

EXPERIMENTAL

Organism. During the initial stages of this work the organism used was the vibrio 01 isolated by Happold & Key (1932); this organism is very similar in morphology and growth characteristics to *Vibrio cuneata* isolated by Gray & Thornton (1928). When the initial stages in the oxidation of the benzene ring became clear, it was deemed desirable to determine how general the metabolic pathway was, by employing the other phenol decomposing bacteria. The following organisms described by Gray & Thornton (1928), and obtained from the National Collection of Type Cultures were used for this purpose: *Mycobacterium crystallophagum* (2566), *Micrococcus sphaeroides* (2559), *Vibrio cuneata* (2583), *Pseudomonas rathonis* (2577) *and Bacillus closteroides* (1584).

Media. The following mineral salt medium has been used throughout: $(NH_4)_2SO_4$ 1·0 g.; $MgCO_3$ 0·5 g.; K_2HPO_4 0·5 g.; $CaCl_2$ 0·1 g.; NaCl 0·1 g.; $FeCl_3$ 0·01 g. dissolved in distilled water (1 l.). The pH was adjusted to 7·2, and the substrate concentration was 50–100 mg./100 ml. medium.

When phenol was used as the metabolite, the concentration used was 50 mg./100 ml. medium; higher concentrations prolonged the initial lag phase in growth, over 100 mg./100 ml. medium being definitely toxic to the organism. Much higher concentrations of benzoic acid, however, were tolerated by the organism, but the usual concentration used was 100 mg. sodium benzoate/100 ml. medium.

Isolation of phenol-utilizing organisms. Soil or faeces (0·5 g.) was inoculated into 100 ml. of sterile medium, contained in tubes that could be aerated with a current of sterile air. Almost invariably growth occurred within a few days, and transplants were made into fresh aeration tubes containing the same medium. After obtaining cultures by this elective method, sub-cultures were made on to nutrient agar plates containing 0·05 g. phenol/100 ml. of nutrient agar. Representative colonies were inoculated into phenol liquid media, and only those organisms that could grow under these conditions, with phenol as the sole organic carbon source, were used for further experiments. The cultures were usually incubated at 35°, but for large scale work at room temperature.

Manometric methods. The oxygen consumption of cell suspensions was measured in the usual way in Warburg manometers at 37° in the presence of 0·1 M-phosphate buffer at pH 6·8. Conical cups (about 20 ml. capacity) with a central well were used; substrates were added from the side arm; O_2 uptake, and CO_2 output were measured. The approximate volume of well-centrifuged bacterial cells used in the respiration experiments was 0·2 ml./manometer cup.

Estimations. Phenol was estimated by the bromination method of Redman, Weith & Brock (1913), and also by the colorimetric method of Folin & Ciocalteau (1927). Catechol substances were estimated by the molybdate colorimetric method of Rae (1930).

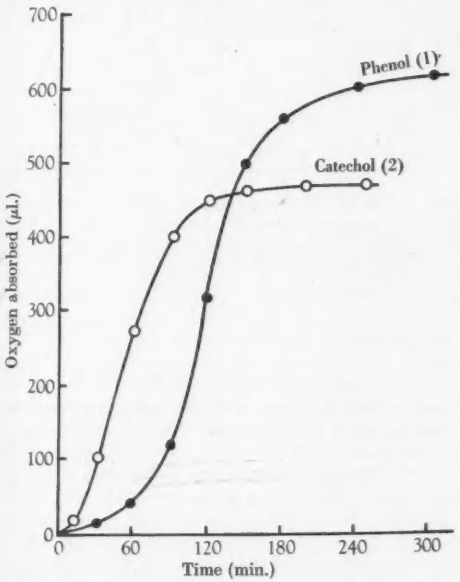

Fig. 1. Oxygen uptake curves with phenol (1) and catechol (2) as substrates using a suspension of the vibrio 01.

Contents of manometer cups, 1 ml. washed suspension (0·2 ml. packed cells) in 0·1 M-phosphate buffer, pH 6·8, and 1 ml. (0·5 mg.) of substrate solution in 0·1 M-phosphate buffer, pH 6·8. Gas phase, air; temperature of bath, 37°.

Separation of mono- and dihydroxyphenolic compounds. A method found extremely useful for the separation of mono- and dihydroxyphenol derivatives, and which has been widely employed during the present investigation, is based on the following observations. Monohydroxy derivatives of benzene, e.g. phenol, *p*-cresol, *p*-hydroxybenzoic acid, and tyrosine, do not give an insoluble lead compound with lead acetate in neutral solution. Dihydroxy derivatives, e.g. catechol, homocatechol, 3:4-dihydroxybenzoic acid, dopa, and adrenaline, in neutral or slightly acid solution with neutral lead acetate give an insoluble lead compound immediately. This behaviour can be made the basis of the quantitative separation of these two types of substances.

Respirometric experiments. The vibrio 01 was grown in the phenol mineral salts medium. In 4 days, under continuous aeration of the culture, at room temperature, heavy growth was obtained. The bacterial cells were centrifuged aseptically, and washed twice by resuspension in distilled water. They were finally suspended in 0·1 M-phosphate buffer, pH 6·8, in a dilution of 2·0 ml. packed cells/10 ml. One ml. of this suspension was used/manometer cup, whilst the side arm contained 1 ml. substrate solution (0·5 mg.) in the same phosphate buffer, making a final volume of 2·0 ml./respirometer cup. Oxygen uptake and CO_2 output were measured at 37°.

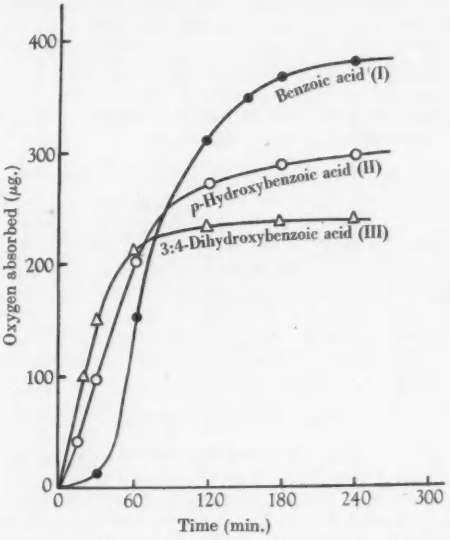

Fig. 2. Oxygen uptake with benzoic acid (I), *p*-hydroxybenzoic acid (II), and 3:4-dihydroxybenzoic acid (III) as substrates, using a suspension of the vibrio 01.

Contents of manometer cups, 1 ml. cell suspension in 0·1 M-phosphate buffer, pH 6·8, and 1 ml. (0·5 mg.) of substrate solution in 0·1 M-phosphate buffer, pH 6·8. Air; 37°.

RESULTS

The curves of O_2 uptake during the course of the oxidation of phenol and catechol can be seen in Fig. 1; for benzoic *p*-hydroxybenzoic and 3:4-dihydroxybenzoic acids in Fig. 2. The resting respiration of the bacterial cells alone have been subtracted, but in no case was this appreciable.

A definite latent period is evident with benzoate and the monohydroxy compounds, but this is appreciably reduced in the case of the dihydroxy compounds. After about 2–3 hr. the initial fairly rapid oxidation rate diminishes considerably, and then subsequently a small O_2 uptake continues for a much longer period, at a rate of approximately

10 μl./hr. This probably indicates that the intermediates accumulating at the end of the rapid oxidation phase, are slowly oxidized further.

The gaseous exchange has been computed with reference to the end of the rapid oxidation stage, and is summarized in Table 1.

Table 1. *Substrates oxidized by suspension of cells of vibrio* 01, O_2 *consumption and* CO_2 *output*

Substrate (0·5 mg.)	O_2 uptake (μl.)	Atoms O/ molecule substrate	CO_2 output (μl.)	Molecule CO_2/ molecule substrate
Phenol	610	10	470	4
Catechol	460	9	400	4
Benzoic acid	370	8	365	4
p-Hydroxybenzoic acid	290	7	325	4
3:4-Dihydroxybenzoic acid	230	6	290	4

The fact that catechol takes up one oxygen atom/molecule of substrate less than phenol is strong evidence that it is the first oxidation product of the latter. Similarly it may be tentatively deduced that the oxidation of benzoic acid proceeds via the *m*- or *p*-hydroxy compound to protocatechuic acid, since salicylic acid is not oxidized by the bacterial suspension. These views were confirmed later by the actual isolation of these intermediate compounds in large-scale culture experiments.

The utilization of phenol

On seeding the medium containing phenol (50 mg./100 ml.) with the vibrio 01 and aerating with sterile air at room temperature, growth is usually visible after 2 days, becoming profuse on the third day. At neutral pH hardly any pigmentation is visible through the dense, opaque bacterial culture. Previous experience of the course of the biological oxidation of phenol (e.g. by the monophenolase, tyrosinase) coupled with results obtained by feeding experiments with animals, made it appear probable that the introduction of more hydroxy groups might be a preliminary step to the disruption of the aromatic ring. A young culture of the phenol organism, as above, when spun to remove bacterial cells, gave a distinct qualitative test for 'catechol' substances, when tested by the delicate tungstate or molybdate colour reaction.* The rate of disappearance of phenol in a culture, and the formation of 'catechol' substance, is given quantitatively in Fig. 3. As will be seen, after the beginning of growth, phenol disappears from the medium fairly rapidly, the intermediate giving the 'catechol' reaction having a transient existence. Phenol has completely disappeared from the culture at room temperature, within 7 days.

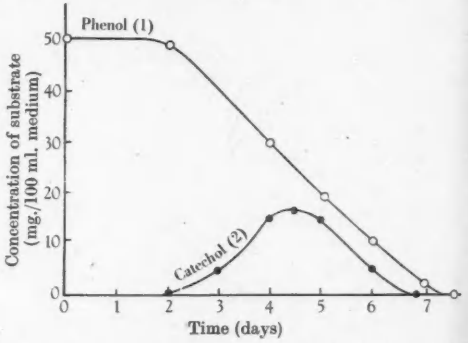

Fig. 3. The rate of disappearance of phenol (1) and the formation of catechol (2) in a phenol culture medium of the vibrio 01.

Initial concentration of phenol 0·5 mg./ml. of medium; medium inoculated by loop, aerated with sterile air, at room temperature (approximately 18°).

Isolation of the 'catechol' intermediate

A phenol culture (10 l., 5 g. phenol), was carefully observed until the 'catechol' reaction was intense. The whole culture fluid was then acidified to pH 3 with H_2SO_4, and filtered through a thin layer of kieselguhr, giving a clear filtrate. This was extracted with ether (3 times) until the mother liquid failed to give the colour reaction. The ether extract was dried with Na_2SO_4 and evaporated to dryness giving an oily residue, which gave an intense 'catechol' reaction. It was taken up in water (50 ml.), filtered, and excess lead acetate solution (20 %) added. A white precipitate immediately separated, which was centrifuged, and washed with distilled water. The precipitate was resuspended in distilled water (20 ml.), and decomposed by H_2S, the PbS filtered off, and the aqueous filtrate re-extracted with ether (3 times). Drying the combined ether extracts with Na_2SO_4 and evaporation of the ether, left a small residue (300 mg.) which crystallized on standing in the cold. It was recrystallized from light petroleum, and then showed m.p. 104°. It afforded a dibenzoyl derivative, m.p. 84° alone and on admixture with authentic dibenzoylcatechol.

The production of o-benzoquinone

A phenol culture (5 l., 2·5 g. phenol) was aerated until growth was visible, and phenol had started disappearing. Freshly distilled aniline (1·0 g.) was

* *Colour test for catechol substances.* The test solution (1 ml.) is made neutral, 1 ml. of a 10 % sodium tungstate or sodium molybdate solution is then added, followed respectively with 0·5 ml. HCl (0·5 N), and 1·0 ml. of a solution of sodium nitrite (0·5 %). A yellow colour immediately develops if 'catechol' substances are present. On addition of 1·0 ml. of NaOH (0·5 N), the yellow colour becomes cherry red.

then added to the culture, and slow aeration continued. Within a few hours, the culture developed a reddish haze, and after a further 2 days, a fine red precipitate was deposited on the sides of the culture vessel. The culture was made distinctly acid to Congo red with HCl, the reddish precipitate filtered off and washed with distilled water. The precipitate was purified by solution in ether, which was filtered and evaporated to dryness. The residue was recrystallized from light petroleum affording bright red needles, m.p. 193°, of dianilino-*o*-benzoquinone and on admixture with an authentic specimen the m.p. was not depressed. (Found: N, 9·63; calc. for $C_{18}H_{14}N_2O_2$: N, 9·66 %.)

Utilization of benzoic acid: the isolation of 3:4-dihydroxybenzoic acid

The vibrio 01, in common with most of the other types of organisms mentioned previously, also grew well in the inorganic salt medium to which benzoic acid had been added as the sole source of organic carbon. This substrate was considerably less toxic than phenol to the bacteria, and was normally used at a concentration of 0·1 g. sodium benzoate/100 ml. medium.

Benzoate culture medium (5 l., 5 g. sodium benzoate) was inoculated, and aerated with sterile air at room temperature in the usual manner. Growth was noticeable after 1 day and was very heavy on the second day. After this period, the sensitive colour test for 'catechol' substances was definitely positive. When this test was at its maximum, the whole culture was made definitely acid to Congo red with H_2SO_4, and filtered through kieselguhr. The clear filtrate was exhaustively extracted with ether, and the combined ether extracts washed with distilled water, followed by excess of a 20 % neutral lead acetate solution. There appeared immediately in the aqueous layer a white precipitate which was filtered off, well washed with water, and decomposed with H_2S. After filtration of the PbS, the aqueous solution was re-extracted with ether, the ether solution dried with Na_2SO_4, and evaporated to dryness. A white solid remained (0·5 g.) which was recrystallized from ethanol-water mixture, giving white needles of m.p. 195° (alone or mixed with authentic 3:4-dihydroxybenzoic acid). It afforded a diacetyl derivative, m.p. 153°.

Both *m*- and *p*-hydroxybenzoic acid, when employed as substrates, gave rise to protocatechuic acid from the bacterial culture, but the organism was without action on salicylic acid.

Attempts to isolate a derivative of the 3:4-quinone of protocatechuic acid (e.g. a dianilino, or sulphone derivative) were unsuccessful.

Other intermediate compounds formed by the bacterial oxidation of phenol and benzoic acid

The substance responsible for the 'Rothera reaction'. Very early on in the investigation of the metabolic products resulting from the bacterial oxidation of phenol or benzoic acid, it was noticed that at a certain stage the culture fluid gave a very definite Rothera reaction. This colour reaction usually began to appear after the 'catechol' test had become negative. At this stage, the culture filtrate also gave definite positive results in Gerhardt's test (Bordeaux red with $FeCl_3$ solution) and Hurtley's reaction (to 10 ml. culture fluid, 2 ml. conc. HCl were added, followed by 1 ml. of $NaNO_2$ solution (1 %); after 2 min., 15 ml. conc. ammonia (sp.gr. 0·880) were added, followed by 5 ml. of a ferrous sulphate solution (10 %). A violet coloration was produced.)

It was originally thought that these colour reactions were due to certain intermediates formed after fission of the aromatic ring, most probably either acetoacetic acid or acetone. However, as these tests are not likely to be specific, and only indicative of certain particular chemical groupings, the matter was further investigated.

A phenol culture which had reached the stage at which a good Rothera reaction was given, was filtered through kieselguhr, and the clear filtrate divided into three portions as follows:

(i) 100 ml. filtrate, made acid to Congo red with H_2SO_4.

(ii) 100 ml. filtrate made alkaline to litmus with NaOH.

(iii) 100 ml. filtrate made alkaline to litmus with $NaHCO_3$.

All of these solutions were separately extracted with ether (acetone free) repeatedly (10 times), and the residual aqueous phase tested with Rothera's reagents. The first solution (i) now gave a much weaker reaction than a control kept at this pH, whilst solutions (ii) and (iii) gave the reaction practically unchanged in intensity. It is evident, therefore, that the unknown intermediate must be of an acidic nature, but even so it is extracted with ether only with difficulty from the aqueous phase.

A comparison with acetoacetic acid and acetone was made as follows. Acetoacetic acid was prepared by the method of Shaffer (1921), and an acidic (to Congo red) aqueous solution adjusted so that the intensity of the Rothera reaction given by it was approximately equal to that of an acidified phenol culture filtrate. Similarly an aqueous acetone solution was adjusted in an identical manner. Upon repeated extraction of the three solutions in parallel, with ether, the following results were obtained (Table 2).

Table 2. *Relative ease of extractability of the 'Rothera substance', acetoacetic acid, and acetone*

Solution	Number of ether extractions	Rothera reaction on mother liquor
Acetoacetic acid	3	Negative
Acetone	3	Negative
Phenol culture filtrate	3	Positive—Rothera test slightly less
Phenol culture filtrate	10	Very pronounced diminution in the Rothera reaction

Close comparison of the Rothera colour reaction given by the bacterial filtrate, with substances containing the S—S linkage like glutathione, or sulphides, show very marked differences between the nature of the colour, its development rate, and persistence, leading to the view that it is not a substance of this nature. The above evidence also seems to exclude identity with acetoacetic acid, but suggests rather that we are dealing with an unstable ketoacid with some similar properties. Further work is in progress with the object of identifying this interesting intermediate of the fission of the benzene ring.

Isolation of formic acid. A phenol culture (2 l. containing 1 g. phenol) was allowed to proceed until the 'catechol' reaction was negative, and all the phenol had disappeared, according to the Folin method of estimation. The cells were removed by filtration through a large Berkefeld candle, and the clear filtrate made distinctly acid to Congo red. To determine the amount of volatile fatty acids, the filtrate was boiled for 15 min., the distillate being collected in 25 ml. of 0·1 N-NaOH. Two l. of culture filtrate gave a volatile fatty acid fraction equivalent to 10 ml. of 0·1 N-NaOH. The distillate gave the following reactions characteristic of a very weak solution of sodium formate: (i) a red colour with $FeCl_3$; (ii) it reduced ammoniacal silver nitrate; (iii) it decolorized acid $KMnO_4$; (iv) partition of the copper salts between *iso*amyl alcohol and water gave a distinct coloration in the water layer but none in the alcohol, indicating the absence of appreciable amounts of propionic and butyric acids.

As the quantities in the above experiments were small it was repeated, with 20 l. of phenol culture, the distillate being collected in 50 ml. N-NaOH solution. This solution was extracted with ether, then made definitely acid to Congo red, and re-extracted several times with ether. These latter ether extracts were combined, dried with Na_2SO_4, and evaporated to dryness. A small oily residue remained, with a pungent odour (0·5 g.). *p*-Toluidine (2 g.) was added to this residue, and the mixture heated in a bath at 220° for 1 hr., cooled and dilute HCl added. The precipitate which separated was filtered, washed thoroughly with water, and re-crystallized from glacial acetic acid; its m.p. was then 53° alone or mixed with the *p*-toluidide of formic acid.

The enzymatic mechanism in the bacterial oxidation of aromatic compounds

The biological oxidation of phenolic compounds has been the subject of extensive investigation, and fairly well defined enzyme systems are known which participate in one or other of the intermediate stages. Thus, the oxidation of monohydroxy to dihydroxy-phenol compounds is catalyzed by only one well defined enzyme, the copper-containing mono-phenolase, tyrosinase. This enzyme, however, has no action on benzoic acid. Oxidation of a dihydroxy-phenol derivative to the corresponding quinone is catalyzed by a variety of seemingly different enzymes, viz. (*a*) The copper-protein enzymes, e.g. tyrosinase, laccase, polyphenol oxidase and dopa (3:4-dihydroxyphenylalanine) oxidase. (*b*) The iron-protein enzymes, e.g. peroxidase, or the cyto-chrome system.

Molecular or 'active' oxygen is required for all these enzymatic oxidations. In no case has it been clearly demonstrated that a hydrogen acceptor other than molecular oxygen will suffice.

According to the evidence presented in this work, the course of the bacterial oxidation of phenol and benzoic acid can be represented as shown in the diagram on p. 379.

Action of bacterial cell suspensions on tyrosine and dopa. It is known that dopa is oxidized to melanin much more readily than tyrosine, by tyrosinase (Evans & Raper, 1937). In view of the pathway followed by phenol and benzoic acid when metabolized by these micro-organisms, it was of considerable interest to study their effect on tyrosine and dopa. A cell suspension (approximately 2 ml. packed cells/10 ml.) of the phenol-splitting bacteria was prepared by centrifugation of a benzoic acid medium culture. The following five aeration tubes were prepared: (1) tyrosine 25 mg. in 50 ml. 0·1 M-phosphate buffer, pH 6·6; bacterial suspension (10 ml.). (2) Similar to (1) but with a boiled bacterial suspension. (3) Dopa 25 mg. in 50 ml. 0·1 M-phosphate buffer, pH 6·5; bacterial suspension (10 ml.). (4) Similar to (3) but with a boiled bacterial suspension. (5) Control tube: cell suspension (10 ml.) alone.

After the addition of a drop of chloroform to each tube they were aerated with NH_3-free sterile air at 37° for 24 hr. At the end of this period samples were taken for the estimation of the amount of substrate still remaining, the residual reaction mixtures being made alkaline with NaOH; any

NH_3 formed was aspirated into 25 ml. of 0·1 N-H_2SO_4 solution. The results are summarized in Table 3.

Table 3. *Action of cell-suspension of vibrio* 01 *on tyrosine and dopa*

Aeration tube contents	Development of pigment	NH_2 liberated (mg.)	Substrate remaining after 24 hr. (%)
(1) Tyrosine (25 mg.) plus cells	Nil	2·0	10
(2) Tyrosine (25 mg.) plus boiled cells	Nil	0·25	98
(3) Dopa (25 mg.) plus cells	Heavy black melanin	0·8	Nil
(4) Dopa (25 mg.) plus boiled cells	Slight melanin production	0·2	90
(5) Control tube cell suspension	Nil	0·15	Nil

As will be seen from Table 3, tyrosine is almost completely deaminated by the cell suspension and disappears from the reaction mixture, with no pigment formation. Dopa, on the other hand is oxidized to black melanin to a considerable extent, undergoing less than 50% deamination. With tyrosine and the bacterial cells, the essential side-chain structure, which, in the oxidation of this substrate with tyrosinase leads to indole substances and eventually to melanin, is preferentially attacked. In consequence, deamination and oxidation of the alanine side chain occurs prior to the oxidation of the aromatic nucleus. In the case of dopa it seems probable that both mechanisms are operative. The non-production of the characteristic pigments of the tyrosine-tyrosinase reaction when these bacteria oxidize tyrosine, should not therefore be taken as evidence of the absence of the monophenolase from the bacterial cells.

The anaerobic oxidation of phenolic compounds by the bacterial suspensions. The phenol-decomposing bacteria do not grow anaerobically, neither is there any appreciable utilization of phenol by a bacterial suspension in absence of oxygen. In view, however, of the clear evidence of the production of *o*-benzoquinone from catechol by the organisms, it was of interest to investigate this dehydrogenation reaction further. It will be recalled that oxygen cannot be replaced by any other hydrogen acceptor when the aerobic oxidases are employed to catalyze this oxidation.

The Thunberg-Keilin technique was used, and a fresh bacterial suspension which had been actively oxidizing phenol prepared by centrifugation followed by thorough washing with distilled water. Reagents were used at the following final concentrations: methylene blue, 0·02%; substrate, 0·1% in 0·1 M-phosphate buffer, pH 6·8 (both these solutions were placed in the side arm of the Thunberg-Keilin tubes); and bacterial suspension, 1 ml. packed cells diluted to 10 ml. The tubes were kept in a bath at 37°, evacuated by Hyvac pump, filled with nitrogen (oxygen free) and re-evacuated twice. The times required for the complete decolorization of the methylene blue were observed, and are recorded in Table 4.

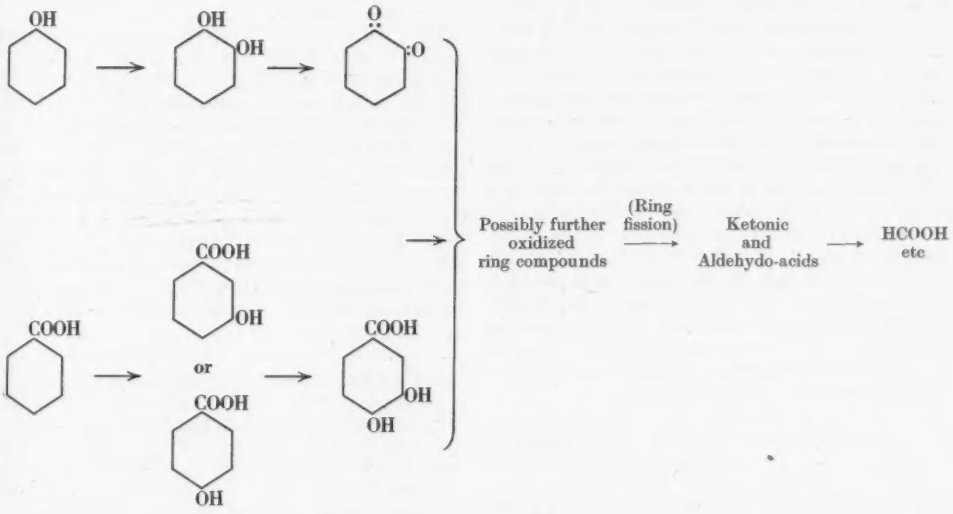

Table 4. *The action of cell-suspensions of vibrio* 01 *on methylene blue and various substrates under anaerobic conditions*

(All tubes contained methylene blue (0·2%), 1 ml. Temp.: 37°. Substrate concentrations: 10 mg./ml.)

Tube no.	Substrate	Cell suspension (10 ml.)	Time for complete decolorization of methylene blue (0·2%)
1	Phenol (1 ml.)	10 ml.	No decolorization in 3 hr.
2	Phenol (1 ml.)	10 ml. (boiled)	No decolorization
3	Catechol (1 ml.)	10 ml.	30 min.
4	Catechol (1 ml.)	10 ml. (boiled)	No decolorization
5	Benzoic acid (1 ml.)	10 ml.	No decolorization
6	Benzoic acid (1 ml.)	10 ml. (boiled)	No decolorization
7	3:4-Dihydroxybenzoic acid	10 ml.	25 min.
8	3:4-Dihydroxybenzoic acid	10 ml. (boiled)	No decolorization

From Table 4 we observe the highly interesting result of an apparent 'catechol dehydrogenase' activity. As far as the author is aware, this represents the first indication of the possibility of a dehydrogenase system being involved in the biological oxidation of *o*-dihydroxy phenols to their respective quinones. It is not known, as yet, which group of dehydrogenating enzymes is responsible for the activation of the catechol derivatives, nor is it clear what is the nature of the intermediate hydrogen acceptors through which it reacts with oxygen.

A heavy suspension of the organism was prepared and investigated for absorption bands by the Hartridge reversion spectroscope. Definite bands were visible in the following regions: (*a*) 530–535 mμ; (*b*) 550–565 mμ; (*c*) 580–585 mμ. It is difficult, at this stage, to ascertain the role of cytochrome systems in the bacterial oxidation of benzene ring compounds which lead to nuclear fission.

Substrates capable of acting as sole metabolites in a simple mineral salt medium by the vibrio 01

Of the organisms described by Gray & Thornton (1928) which were capable of utilizing aromatic compounds in a simple salt medium, a certain amount of specificity towards metabolites was encountered; thus, whilst almost all oxidized phenol, only some were able to grow in the medium containing benzene, toluene or naphthalene. With the vibrio 01 which was the main organism used in this investigation, it is interesting to observe its general oxidative capacity. Table 5 gives a list of the substrates which when present in the salt medium are able to support growth (indicated by a positive sign), compared with related compounds which were found inadequate for growth (indicated by a negative sign).

Table 5. *The effect of various substances as sole metabolites for the vibrio* 01, *in a salt medium at* 30°

(All substrates were used at a final concentration of 0·01 g./100 ml. of the mineral salt medium.)

	Substrates	Growth
Phenols	Phenol	+
	Catechol	+
	p-Cresol	−
	Resorcinol	−
Substituted benzoic acids	Benzoic acid	+
	m-Hydroxybenzoic acid	+
	p-Hydroxybenzoic acid	+
	o-Hydroxybenzoic acid	−
	3:4-Dihydroxybenzoic acid	+
	Phthalic acid	−
	Terephthalic acid	−
	p-Aminobenzoic acid	−
Phenolic amino-acids	Tyrosine	+
	3:4-Dihydroxyphenylalanine	+
Fatty acids (sodium salts)	Acetate	+
	Lactate	+
	Oxalate	−
	Citrate	+
	Pyruvate	±
	Succinate	+
	Formate	±
	Fumarate	+
	trans-trans-Muconate	−
	Adipate	+
	Malonate	−
	Malate	+
	dl-Tartrate	−

Bernheim (1942) compared the oxidative capacities of the tubercle bacillus (bovine) with a non-pathogenic mycobacterium, with regard to benzoic acid and related substances. Table 6 reproduces his results as regards specificity of the substrates oxidized.

Table 6. *The action of cell suspensions of a tubercle bacillus (bovine) and a non-pathogenic mycobacterium on various substrates (Bernheim,* 1942)

	Organism	
Substrate	T.B. (bovine)	Non-pathogen (*Mycobacterium*)
Benzoate	+	+
o-Hydroxybenzoate	+	−
m-Hydroxybenzoate	−	+
p-Hydroxybenzoate	−	+
Tyrosine	−	+
Phenylalanine and other amino-acids	−	−
Phenol	−	+

It is evident that Bernheim's non-pathogenic *Mycobacterium* behaves in much the same way as the vibrio 01.

DISCUSSION

The fate of aromatic compounds in living organisms has long been a topic in intermediate metabolic studies. Thus, Tauber (1878) brought forward some quantitative data of the amount of phenol disposed of by oxidation, as distinct from detoxification mechanisms *in vivo*. He estimated that in the rabbit, 25–50% of a sublethal dose was broken down by the animal and completely oxidized; traces of pyrocatechol and hydroquinone were isolated from the urine. Deichmann (1944) reinvestigated this problem, also in the rabbit; he found that when the phenol is administered in sublethal doses, detoxication by conjugation is the major route (77%) by which the compound is eliminated from the body, about 20% being oxidized completely. When, however, the dose is approaching the lethal level, and the excretion of the conjugated product is diminished, as a result of injury to the kidneys, the destruction of the compound by oxidation is the predominant mechanism. Little light has been thrown on the oxidation mechanism or the intermediate metabolites by these studies.

Embden & Balder (1913), in a study of the fate of phenylalanine and tyrosine in the mammalian liver, brought forward evidence of acetoacetic acid formation, but nothing definite was established as to the mechanism of ring cleavage. The studies of Moss & Schoenheimer (1940) using the tracer technique, have made it practically certain that in the rat tyrosine is formed from phenylalanine. Bernheim & Bernheim (1944) have located where this oxidation might take place by their demonstration that rat and guinea-pig liver slices oxidize phenylalanine to give a product which is almost certainly tyrosine. Evidence for the nuclear oxidation of tyrosine is forthcoming from a variety of studies, mainly in connexion with disorders of metabolism; thus an inborn error of metabolism has been described by Medes (1932) called 'tyrosinosis', whence the administration of tyrosine by mouth led to the excretion of 3:4-dihydroxyphenylalanine (dopa) in the urine. The reported formation of small amounts of adrenaline when a tyramine solution is incubated with thin slices of the suprarenal gland by Schuler & Wiedemann (1935), also indicates the presence in mammalian tissues of enzymes capable of the oxidation of the aromatic nucleus.

Turning now to the evidence forthcoming from metabolic work on benzene itself, Drummond & Finar (1938) confirm previous workers, that this substance gives rise to *trans-trans* muconic acid when fed to rabbits. The *cis-cis* acid would be the one directly derived from the opening of the benzene ring, but they suggest that this stereoisomeride is converted into the *trans-trans* acid in the presence of other products formed when benzene is metabolized. Boeseken & Englebeits (1932), however, incline to the view that the *trans-trans* muconic acid is an indirect product of metabolism. Drummond & Finar failed to isolate muconic acid when phenol, catechol or tyrosine was given to rabbits instead of benzene. It will be noted that the phenol utilizing vibrio 01 is unable to metabolize *trans-trans* muconic acid in an inorganic medium.

The study of the oxidative disruption of benzene ring compounds by micro-organisms is, in many ways, a more attractive experimental approach for investigating the mechanism of the fission of the aromatic nucleus in biological oxidation.

Furthermore, these studies are of industrial significance in so far as the purification of chemical waste effluents containing phenolic substances, for example, probably depends for its success on the activities of these types of micro-organism. Also, in the agricultural field, these organisms may well be concerned in the putrefaction of organic matter and in preventing the harmful accumulation of toxic aromatic substances in the soil.

SUMMARY

1. The utilization of phenol and benzoic acid by certain micro-organisms isolated from the soil, sewage and faeces, has been investigated.

2. It has been shown that, using a simple mineral salt medium, with phenol or benzoic acid as the sole source of organic carbon these organisms will grow and completely metabolize these substrates.

3. The following gaseous exchange occurs during the oxidation of these substrates by a washed bacterial suspension of the vibrio 01: (*a*) phenol (1 molecule) utilizes 10 atoms of O, (*b*) catechol (1 molecule), 9 atoms, (*c*) benzoic acid, 8 atoms, (*d*) *p*-hydroxybenzoic acid, 7 atoms and (*e*) 3:4-dihydroxybenzoic acid, 6 atoms/molecule. All liberate 4 molecules CO_2.

4. The fact that catechol takes up one atom of oxygen less than phenol indicates that it is the first oxidation product of the latter. This was confirmed by direct isolation from a bacterial culture starting with phenol as substrate.

5. Similarly, the observation that *m*- or *p*-hydroxybenzoic acids take up one atom of oxygen less than benzoic acid, indicates that one or the other, is the first oxidation product of the latter.

6. Since 3:4-dihydroxybenzoic acid absorbs one oxygen atom less than either *p*- or *m*-hydroxybenzoic acid, it is likely to be the direct oxidation product. This was shown to be the case by actual isolation of protocatechuic acid from the bacterial culture starting with benzoic acid as substrate.

7. During the bacterial oxidation of phenol or catechol, it has been shown that *o*-benzoquinone is produced, by the isolation of a di-anilino derivative.

8. Another intermediate compound, having a transient existence, gives the Rothera reaction. This substance has not been characterized, but evidence is presented which indicates that it may be a keto-acid, although not identical with acetoacetic acid.

9. Formic acid has been isolated from a bacterial culture in which the phenol or benzoic acid had disappeared.

10. It is shown that in the action of a bacterial suspension of vibrio 01 on the two amino-acids, tyrosine and 3:4-dihydroxyphenylalanine, two oxidative mechanisms may operate: deamination leading to oxidation of the side chain, or nuclear oxidation leading to fission of the ring.

11. Evidence is presented for the occurrence of a 'catechol dehydrogenase system' in cell suspensions of these organisms.

12. Our knowledge of the oxidation of the benzene ring within living organisms and the possible role of these micro-organisms in the prevention of aromatic compounds from accumulating in toxic amounts in the soil is discussed.

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Amino-Acids and Proteins in Haemoglobin Formation

I. TRYPTOPHAN*

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Knowledge of protein and amino-acid metabolism is confined almost entirely to those aspects concerned with degradation and excretion. Of the synthetic processes in which amino-acids are utilized little is known. One of the most important of such syntheses is that of haemoglobin, the new formation of which in the adult human being is estimated at about 25 g./day. Red blood cell destruction followed by the irreversible decomposition of the haemoglobin and excretion of part of the resulting products in the form of urinary and faecal pigments takes place continuously in the normal animal and the nitrogenous material thus lost has to be replaced by dietary protein. We have at present, however, very little knowledge of the amino-acids essential for the performance of this function.

Tryptophan, which has an obvious chemical relationship to blood porphyrin, is the only amino-acid which has been extensively investigated from this point of view. The efficacy of tryptophan in the treatment of experimental anaemia was first noted by Hirasawa (1922) and similar results were reported by Okagawa & Tatsui (1931). Fontés & Thivolle (1930) claim to have produced anaemia in rats kept on a tryptophan deficient diet for a period of 10 weeks, and further, to have rendered normal

* A preliminary communication was published in *Current Science*, **12**, 326 (1943).

rabbits and dogs hyperaemic by injections of tryptophan or a mixture of tryptophan and histidine. A mixture of these two amino-acids was claimed by these authors to have also a curative effect in certain types of human anaemia. Alcock (1933), however, was unable to produce anaemia in animals by depriving them of tryptophan, nor could he demonstrate any beneficial effect on administering tryptophan to rats suffering from milk anaemia. Tochowicz (1936) also suggests that tryptophan plays no important role in blood regeneration either in haemorrhagic or pernicious anaemia. Other authors (Cuthbertson, Fleming & Stevenson, 1931; Dominici & Penati, 1931) have confirmed the negative results in pernicious anaemia. But in experimental animals Hamada (1936) reproduced the observations of Fontés & Thivolle, and succeeded in making young rats and mice anaemic by keeping them on a tryptophan deficient diet. Similar results were obtained by Albanese, Holt, Kajdi & Frankston (1943) who found that a diet deficient in tryptophan brings about a reduction of plasma proteins and haemoglobin in the rat.

While the position with regard to tryptophan is thus still controversial, about the role of other amino-acids we have even less information. Whipple & Robscheit-Robbins (1937) have shown that in dogs, made anaemic by bleeding, haemoglobin production is usually, though not invariably, increased by addition of histidine or phenylalanine to a basal bread ration of unspecified amino-acid composition. The findings of Harris, Neuberger & Sanger (1943) that lysine deficiency in rats produces a mild anaemia have been recently confirmed by Gillespie, Neuberger & Webster (1945). On the other hand, in experiments in which rats were made anaemic by the ingestion of a low protein diet, Orten & Orten (1945) found no increase in haemoglobin on supplementation with any one of the ten essential amino-acids or with glycine, cystine, glutamic acid, proline or tyrosine. From experiments on rats kept on human or beef globin as the sole source of protein with and without supplements of isoleucine Orten, Bourque & Orten (1945) conclude that isoleucine is essential for haemoglobin formation in the rat. This surprising result implies either that rat globin, unlike human or beef globin, contains isoleucine or that this amino-acid is required for the synthesis of a protein of which it is not a constituent.

These isolated data suggested the need for further research and a systematic investigation was begun in this laboratory of the haemopoietic action of amino-acids and the nutritional value of proteins as assessed from their efficacy to promote haemoglobin regeneration. It was found quite early in our experiments that normal animals were of little use for this purpose as they showed no marked response in their blood picture either to amino-acid additions or deficiencies. On deficient diets the animals usually lost weight but the percentage of red cells and haemoglobin was not usually altered, although it is probable that there was a decrease in blood volume and therefore a fall in the total haemoglobin in the animal as a whole. It therefore became necessary to make the animals anaemic and to study the rate of recovery from the anaemia on administration of the dietary factor under test. Whipple employed dogs made anaemic by bleeding. The time and expense involved in carrying out large numbers of feeding experiments with amino-acids on dogs made it impossible to use this method. Animals fed on milk proteins and suffering from anaemia on account of a mineral deficiency (Pearson, Elvehjem & Hart, 1937) could also not be considered suitable experimental material for the present purpose. A very convenient means of producing anaemia in experimental animals was found in the use of phenylhydrazine. A single intraperitoneal injection of phenylhydrazine administered at a dose of 2 mg./100 g. body weight induces in rats a consistently reproducible type of anaemia, the red blood cells being lowered to about 50% and haemoglobin to about 60% of the normal values in the course of 3–4 days after injection (see Yeshoda, 1942). The drug is known to act specifically by destroying mature red cells and to have no other toxic effects in the dosages required (Long, 1926*a*, *b*; Stealy & Sumerlin, 1944). In particular, it has no direct effect on the bone marrow or the spleen, and this anaemia is thought to be due entirely to the fact that phenylhydrazine renders red cells susceptible to those processes which normally destroy old erythrocytes (see Sollmann, 1937). The effect therefore is the same as in haemorrhagic anaemia with the possible difference that some of the disintegration products derived from the haemoglobin might be retained in the system and reutilized for new haemoglobin formation. The animals show no other adverse effects due to phenylhydrazine and on a complete diet rapidly recover from the anaemia. The present paper describes experiments on the effect of tryptophan deficiency on the rate of recovery of animals suffering from phenylhydrazine anaemia.

EXPERIMENTAL

The tryptophan-free diet was made up as follows:

	%
Hydrolyzed casein	18
Starch (tapioca)	40
Sugar	20
Butter fat (melted)	17
Salt mixture	5

Each animal also received 2 drops of cod-liver oil and 200 mg. of yeast daily.

The casein was hydrolyzed with sulphuric acid in the usual way and the hydrolysate after removal of acid by means of baryta was evaporated to a thick syrup. This was mixed with the required quantity of starch and dried at a low temperature in a current of air. To the powdery material the remaining constituents of the diet were added and thoroughly mixed. The food was supplied to the animals *ad libitum*. The animals on the tryptophan diet received daily, in addition to the above, 20 mg. of *l*-tryptophan which, mixed with a little melted butter, was given separately. Records of food intake show that consumption ranged from 6–8 g./day, depending upon the body weight of the animal. Rats in the two groups showed no differences in the intake in the first 3 weeks of the experimental period; after that there was a gradual falling off in the food consumption of the deficient group.

The animals were made anaemic by means of a single intraperitoneal injection of 2 mg. phenylhydrazine (1 ml. of 0·2% (w/v) aqueous solution) and put on the tryptophan-free basal diet on the day of injection. From the 4th day 20 mg. of tryptophan/rat/day were given to one group of animals while the control group remained throughout the experimental period on the tryptophan-free diet. Red cell count and haemoglobin determinations were carried out every 3rd or 4th day. Blood was obtained by snipping the tail; the acid haematin method was used for haemoglobin determination.

Table 1. *Recovery of rats from phenylhydrazine anaemia; basal diet without tryptophan*

(R.B.C. values are in millions/mm.^{3}; Hb values in g./100 ml. blood.)
Only four representative experiments are given in this table.

		Days after injection								
Rat no.		0	4	8	12	15	20	25	30	35
33	R.B.C.	6·68	2·80	3·45	4·51	5·07	5·10	5·23	6·18	6·29
	Hb	14·7	8·6	9·3	10·9	11·4	12·3	12·4	13·0	14·0
34	R.B.C.	6·91	2·82	3·60	4·26	4·76	5·31	5·91	6·45	6·52
	Hb	14·7	8·5	8·9	11·1	11·9	12·0	12·5	13·0	14·0
35	R.B.C.	6·99	3·27	3·69	3·99	5·01	4·96	6·12	6·13	6·20
	Hb	14·9	8·3	9·0	11·0	11·5	11·5	13·0	12·5	13·9
36	R.B.C.	6·33	3·19	3·67	4·20	4·91	5·20	5·81	5·89	5·94
	Hb	13·5	8·3	9·0	10·5	10·7	11·3	11·9	12·1	12·6

Table 2. *Recovery of rats from phenylhydrazine anaemia; basal diet + 20 mg. oral tryptophan/day*

(R.B.C. values are in millions/mm.^{3}; Hb values in g./100 ml. blood.)
Only four out of eight experiments are reported in this table.

		Days after injection								
Rat no.		0	4	8	12	15	20	25	30	35
41	R.B.C.	6·61	2·65	4·70	5·97	6·71	6·91	6·89	7·02	7·15
	Hb	14·5	8·6	10·6	12·5	13·9	13·9	15·1	15·0	14·8
42	R.B.C.	6·80	3·02	4·98	5·61	6·52	6·60	7·09	7·14	7·20
	Hb	14·9	8·4	11·0	12·4	14·2	14·7	14·9	14·7	14·9
43	R.B.C.	7·02	2·94	4·54	6·06	6·96	6·99	7·09	6·95	7·08
	Hb	15·1	8·5	10·5	12·0	14·1	14·4	14·2	14·5	14·8
44	R.B.C.	6·42	3·16	4·61	5·61	6·26	6·28	6·71	6·81	6·89
	Hb	13·7	8·3	10·3	11·7	13·0	13·5	13·6	13·4	13·6

Table 3. *Body weights of rats mentioned in Tables* 1 *and* 2

(Body weight is given in g.)
The data are representative of a large number of experiments carried out.

		Days											
Rat no.	Tryptophan (mg./day)	0	7	14	21	28	35	42	49	56	60	64	70
33	20	113	118	120	120	123	128	130	—	—	—	—	—
41	—	118	117	98	94	88	80	75	70	57	54	50	*
34	20	122	126	128	130	131	136	138	—	—	—	—	—
42	—	126	122	118	107	104	101	92	86	80	78	76	75
35	20	126	130	132	134	136	138	138	—	—	—	—	—
43	—	126	124	118	99	98	92	85	79	70	63	60	60
36	20	107	108	110	114	119	125	130	—	—	—	—	—
44	—	107	100	94	88	86	83	72	67	52	*	—	—

* Animal died.

RESULTS

Tables 1 and 2 show clearly the difference in the rates of recovery from anaemia of the groups of animals with and without tryptophan in the diet and are typical of the results of many sets of experiments. The accelerated formation of haemoglobin and red cells in the animals receiving tryptophan is particularly marked in the first 12 days after injection. The weights of the animals are given in Table 3. Throughout the experimental period the animals receiving tryptophan steadily increased in weight while the reverse was the case with the rats on the tryptophan deficient diet.

Injection experiments with tryptophan

Hamada (1936) noted that the effect of tryptophan administered by injection is not the same as when it is given by mouth. Experiments were also carried out therefore in which the animals were given subcutaneous injections of 10 or 20 mg. of tryptophan daily. The R.B.C. and haemoglobin values in comparison with the controls are given in Table 4 and the weights in Table 5. It is obvious that injected tryptophan has the same haemopoietic action as when it is supplied in the diet; but given parenterally the amino-acid has no effect on weight. These results are not necessarily in conflict with those of Du Vigneaud, Sealock & Van Elten (1935) who found that tryptophan administered subcutaneously was utilized for growth, as the above authors carried out their experiments on normal animals while the present observations as well as those of Hamada were made on anaemic animals.

Experiments with normal animals

To ascertain the effect of tryptophan deficiency on normal animals three groups of rats were used,

Table 4. *Recovery of rats from phenylhydrazine anaemia; basal tryptophan-free diet + injected tryptophan*

(R.B.C. values are in millions/mm.³; Hb values in g./100 ml. blood.)

Rat no.	Tryptophan injected (mg./day)		Days after injection: 0	4	7	10	13	16
101	0	R.B.C.	7·51	3·27	4·17	5·02	5·55	5·91
		Hb	14·5	8·3	10·1	10·7	12·3	12·5
102	0	R.B.C.	7·29	3·50	3·56	4·71	5·31	6·01
		Hb	14·3	8·1	9·1	11·9	12·0	13·0
103	0	R.B.C.	7·01	3·91	4·13	4·94	5·05	5·74
		Hb	13·8	8·0	10·1	11·4	12·0	12·2
104	0	R.B.C.	7·53	3·18	4·05	5·00	5·52	5·70
		Hb	15·1	8·1	9·1	12·0	12·3	12·5
105	10	R.B.C.	7·41	3·90	5·15	5·57	6·71	7·22
		Hb	14·2	8·5	11·8	12·3	13·6	14·0
106	10	R.B.C.	7·31	3·80	4·56	5·59	6·91	7·12
		Hb	14·9	8·5	11·0	12·5	13·7	14·2
107	10	R.B.C.	7·20	3·11	5·56	5·86	6·40	7·06
		Hb	13·8	8·3	12·0	13·0	13·5	14·1
108	10	R.B.C.	7·50	3·22	4·81	5·66	6·40	7·35
		Hb	15·2	8·1	11·3	13·0	13·8	14·5
109	20	R.B.C.	7·37	3·47	5·46	6·17	7·19	7·41
		Hb	14·6	8·3	11·8	14·0	14·1	14·8
110	20	R.B.C.	7·29	3·69	5·63	5·90	7·21	7·38
		Hb	14·5	8·5	12·0	13·0	14·5	14·8
111	20	R.B.C.	7·01	8·43	4·91	5·91	6·98	7·15
		Hb	14·0	8·3	12·0	13·1	14·0	14·3
112	20	R.B.C.	7·37	3·44	4·88	5·71	6·69	7·15
		Hb	14·4	8·1	11·7	12·0	13·6	14·3

Table 5. *Body weights of rats mentioned in Table 4*

(Body weight is given in g.)

Rat no.	Tryptophan injected (mg./day)	Days: 0	3	6	9	12	15
101	0	120	117	114	110	104	98
102	0	125	123	118	110	100	96
103	0	117	114	110	106	98	90
104	0	102	98	90	84	80	76
105	10	120	118	114	110	105	100
106	10	122	118	114	110	108	103
107	10	115	115	111	106	103	102
108	10	107	100	94	86	80	77
109	20	119	116	112	106	105	103
110	20	122	118	114	110	106	108
111	20	115	114	112	108	104	100
112	20	98	96	92	90	88	86

one receiving hydrolyzed casein, the other hydrolyzed casein plus tryptophan and the third group casein. Only R.B.C. determinations were made in this experiment. The values in Table 6 show that although the erythrocyte count is slightly in favour of animals receiving tryptophan (either as a supplement to hydrolyzed casein or as whole casein) the differences are not sufficient to make them statistically significant.

DISCUSSION

The experiments recorded make it clear that tryptophan accelerates the recovery of animals suffering from phenylhydrazine anaemia. It will be seen from Table 1 that animals suffering from phenylhydrazine anaemia on a tryptophan-deficient diet take about 30 days to recover from the anaemia. When the diet is supplemented with tryptophan the red cells are restored to normal in about 12 days, and haemoglobin in less than 15 days (Table 2). The statistical examination of the results (Table 8) shows that differences between the groups of animals on the tryptophan-containing and tryptophan-deficient diet are highly significant. With tryptophan administration, the average increases in 12 days of red

Table 6. *Body weights and erythrocyte counts of normal rats on different diets*

(R.B.C. values are in millions/mm.^{3}; body weight in g.)

		Casein		Hydrolyzed casein + tryptophan		Hydrolyzed casein	
Rat no.	Days ...	0	30	0	30	0	30
113	R.B.C.	7·00	8·10	7·06	8·05	6·81	7·40
	Weight	61	94	60	86	69	51
114	R.B.C.	7·06	8·05	7·47	8·10	7·46	7·35
	Weight	62	80	64	90	66	63
115	R.B.C.	7·09	8·10	7·29	8·09	7·07	6·60
	Weight	50	95	53	68	53	40
116	R.B.C.	7·08	7·98	6·61	8·01	6·73	7·60
	Weight	45	100	44	68	44	30

In another experiment six animals were maintained on a tryptophan-free diet for a prolonged period until, in fact, most of them died. In this experiment also no significant decrease was observed either in haemoglobin or in the number of erythrocytes, but the animals lost weight heavily (Table 7).

Table 7. *Body weights and blood picture of rats fed a tryptophan-free diet for a prolonged period*

(R.B.C. values are in millions/mm.^{3}; Hb in g./100 ml. blood; body weight in g.)

		Days									
Rat no.		0	14	28	35	42	49	56	58	64	75
201	R.B.C.	6·99	6·80	6·94	6·88	6·98	6·91	6·81	—	—	—
	Hb	14·0	13·7	13·9	13·8	13·9	13·8	13·8	—	—	—
	Weight	84	70	64	58	50	42	39	39*	—	—
202	R.B.C.	7·20	7·10	6·99	7·02	6·99	6·98	6·99	—	6·91	—
	Hb	14·2	14·1	14·0	14·1	13·9	13·9	13·9	—	14·0	—
	Weight	80	70	61	60	56	52	48	44	43	40*
203	R.B.C.	7·02	7·01	6·98	6·98	6·85	6·86	6·75	—	—	—
	Hb	14·2	14·0	14·1	14·0	13·9	13·8	13·8	—	—	—
	Weight	90	78	70	60	54	51	48	44	40*	—
204	R.B.C.	6·75	6·65	6·80	6·75	6·71	6·68	6·65	—	6·68	—
	Hb	13·9	13·9	14·0	13·9	13·8	13·8	13·8	—	13·8	—
	Weight	102	87	82	70	66	62	58	54	52	50
205	R.B.C.	6·85	6·96	6·88	6·98	6·86	6·91	6·99	—	—	—
	Hb	14·2	14·2	14·0	14·1	13·9	13·9	14·0	—	—	—
	Weight	100	90	84	70	59	50	46	45	43*	—
206	R.B.C.	7·22	7·18	6·98	7·01	7·00	6·99	7·01	—	6·96	—
	Hb	14·3	14·2	14·2	14·2	14·0	13·9	14·0	—	14·0	—
	Weight	108	94	83	73	66	64	53	48	45	40

* Animal died.

Table 8. *Average increase in* R.B.C. *and haemoglobin of rats in first* 12 *days after phenylhydrazine—tryptophan by mouth.* (*Statistical analysis of data from Tables* 1 *and* 2 *and of those from other experiments*)

	Hydrolyzed casein + tryptophan		Hydrolyzed casein		
No. of animals ...	8		8		
	Mean increase x_1	Standard error of mean ϵ_1	Mean increase x_2	Standard error of mean ϵ_2	$\frac{x_1 - x_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$
R.B.C.	3·41	0·1525	1·72	0·1052	9·12
Hb	5·17	0·1608	2·96	0·1056	10·64

Table 9. *Average increase in* R.B.C. *and haemoglobin of rats in first* 12 *days after phenylhydrazine—tryptophan by injection.* (*Statistical analysis of data from Table* 4 *and of those from other experiments*)

	Hydrolyzed casein + 20 mg. tryptophan		Hydrolyzed casein + 10 mg. tryptophan		Hydrolyzed casein			
No. of animals ...	4		4		4			
	Mean increase x_1	Standard error of mean ϵ_1	Mean increase x_2	Standard error of mean ϵ_2	Mean increase x_3	Standard error of mean ϵ_3	$\frac{x_1 - x_3}{\sqrt{(\epsilon_1^2 + \epsilon_3^2)}}$	$\frac{x_2 - x_3}{\sqrt{(\epsilon_2^2 + \epsilon_3^2)}}$
R.B.C.	3·76	0·0586	3·68	0·2103	2·37	0·1841	7·24	4·17
Hb	6·23	0·0983	5·83	0·2201	4·43	0·1476	10·19	5·28

cells and haemoglobin exceed by 100 and 74% respectively the corresponding increases on a tryptophan-deficient diet.

Subcutaneous injection of the amino-acid is as effective as admixture with the diet for the purpose of blood regeneration, the R.B.C. and haemoglobin increasing at about the same rate in the two cases (Tables 4 and 9). In one important respect, however, subcutaneously injected tryptophan differs from oral tryptophan. With the former the animal loses as much weight as on a tryptophan-deficient diet. These findings agree generally with the observations of Hamada (1936). But while Hamada found that injected tryptophan in doses larger than about 75–100 mg./kg. body weight retarded red cell regeneration, no such harmful effect was noted in the present experiments in which tryptophan was injected at two levels of about 90 and 180 mg./kg. body weight.

While these experiments establish the beneficial effect of tryptophan in phenylhydrazine anaemia, its mode of action is not yet clear. The possibility that it neutralizes the toxic effects of phenylhydrazine need not be seriously considered as in these experiments tryptophan administration was started 3 days after injection of phenylhydrazine by which time the anaemia had already reached its peak. Further, in experiments on animals maintained on a diet containing whole casein the intensity of the anaemia was unaffected by the presence of tryptophan at the time when phenylhydrazine was injected (Yeshoda, 1942). It is therefore certain that tryptophan exerts its action not by detoxicating phenylhydrazine and preventing the destruction of erythrocytes but by accelerating the formation of new red cells. This it might achieve either by supplying an essential structural unit for haemoglobin synthesis, or by stimulating the haemopoietic organs. The second explanation was favoured by Alcock (1933), who based this view on two findings: (1) that animals suffering from milk anaemia recover from the anaemia independently of the presence of tryptophan in their diet and (2) that animals on a tryptophan-deficient diet do not develop anaemia. The first observation, which is in contrast to our findings, is probably to be attributed to the unsuitability of the method used for inducing anaemia. In view of the demonstration by Robscheit-Robbins and Whipple (1935) that on a favourable diet large reserve stores of haemoglobin-producing material can accumulate in the body, it is not surprising that Alcock's animals, fed on milk proteins and made anaemic by lack of iron and copper, should show no positive response to the amino-acid precursors required for haemoglobin synthesis.

Alcock's second observation that normal animals on a tryptophan-deficient diet do not develop a severe anaemia agrees with our own results. This cannot, however, be considered an argument against the view that it is an essential precursor of haemoglobin, as animals lost weight on the deficient diet and the tryptophan necessary for haemoglobin formation could have arisen from tissue degradation. It is to be remembered that in an animal which is losing weight there is bound to be a proportionate decrease in blood volume and hence of the haemoglobin content, although the relative composition of the blood elements may show no appreciable alteration. Thus in spite of a more or less constant red cell count and haemoglobin percentage there would be a progressive fall in the total haemoglobin

in the body with a consequent decrease in the demand for the structural elements required for haemoglobin regeneration.

Another fact quoted by Alcock in favour of his hypothesis, that the haemopoietic activity of tryptophan is due to its stimulating action on the blood-forming organs, was the observation made by Matsuoka & Nakao (1931) on rats and guinea-pigs suffering from phenylhydrazine anaemia and later confirmed by Chin Kyu-Sui (1938) on rabbits, that injected methyltryptophan accelerates R.B.C. and haemoglobin formation in the same manner as tryptophan. There is no reason, however, to consider that the presence of the 2-methyl group would prevent the utilization of the pyrrole nucleus in tryptophan for porphyrin synthesis. Either demethylation could take place giving rise to tryptophan or the methyl group might be utilized in the formation of the methine bridge present in porphyrins.

The difference in the effects produced by dietary and injected tryptophan still remains to be explained. It is probably to be attributed to possible differences in the metabolic fate of the amino-acid depending upon its mode of entry into the body. It is known that tryptophan metabolism in the animal follows more than one pathway, the amino-acid being converted into indolepyruvic acid, kynurenic acid and urochrome. It does not appear improbable that subcutaneously injected tryptophan undergoes only those transformations that lead to the formation of blood pigments and for this reason is not utilized for the maintenance of weight or growth. It has also to be pointed out that in the injection experiments that have been carried out with tryptophan as well as with methyltryptophan the quantities used have been of the same order as that derived from the diet. It would be reasonable to assume that much smaller quantities of these substances would be sufficient if this action consisted of a stimulation of the haemopoietic apparatus. But no evidence has been produced to show that such small quantities are in fact effective. In any event Alcock's theory of haemoglobin formation without tryptophan which was later expanded to a theory of protein synthesis without amino-acids (Alcock, 1936) can no longer be taken seriously in the light of recent experiments using amino-acids marked with N^{15} (Schoenheimer, Ratner, Rittenberg & Heidelberger, 1942).

SUMMARY

1. It has been shown that in animals suffering from anaemia produced by phenylhydrazine, regeneration of haemoglobin and red cells is markedly accelerated by dietary tryptophan.

2. Tryptophan given by subcutaneous injection also exercises the same haemopoietic effect, but the amino-acid when administered in this manner is not utilized for maintenance of body weight. The results obtained are highly significant.

3. The mode of action of tryptophan and the contradictory findings of previous authors are discussed in the light of these observations. There is no justification for the view that the beneficial effect of tryptophan in blood regeneration does not depend upon its action as a precursor of structural material for haemoglobin building, but upon its stimulatory action on the haemopoietic organs.

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The Fermentation of Sucrose by *Aerobacter aerogenes*

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The production of 2:3-butylene glycol as a bacterial metabolic product from carbohydrate was first observed by Harden & Walpole (1906) and Walpole (1911), but received comparatively little further attention until the second world war when the formation of this product as an intermediate for butadiene, synthetic rubber and other purposes was investigated in considerable detail. Recent work in Canada and the U.S.A. (Adams & Stanier, 1945; Perlman, 1944; Stanier & Adams, 1944; Ward, Pettijohn & Coghill, 1945), has established the characteristics of three distinct fermentations yielding 2:3-butylene glycol: (*a*) the *Aerobacter aerogenes* fermentation of hexoses yielding a mixture of *d*- and *meso*-isomers of the glycol, in which the latter predominates as the main non-gaseous product, (*b*) the *Aerobacillus polymyxa* fermentation of starches and dilute sugar solutions which yields *l*-2:3-butylene glycol and ethanol, and (*c*) the *Aeromonas hydrophila* fermentation in which an *l*- and *meso*-mixture of 2:3-butylene glycol isomers, ethanol, and lactic acid are the main non-gaseous products. The object of the present investigation was to study the production of *d*- and *meso*-2:3-butylene glycol from *Aerobacter aerogenes* fermentations of sucrose as the basis for a technical method using molasses. The factors influencing rate of fermentation and yield of product have been investigated with a view to decreasing the time necessary for complete fermentation and simplifying the culture medium.

EXPERIMENTAL

Composition of media

Although the early workers used complex nutrients such as peptone as a basis for the culture media it is now well known that *A. aerogenes* grows well on simple media containing citrate or glucose as carbon source and nitrates or ammonium salts as source of nitrogen (Koser, 1924; Lodge & Hinshelwood, 1944). The basal medium used in the present work was based on the formulae of Koser (1924) and of Czapek (as modified by Dox (1910)) and had the following composition:

	g.
Sucrose	100
Dipotassium hydrogen phosphate (K_2HPO_4)	2·5
Ammonium sulphate	4·0
Magnesium sulphate ($MgSO_4.7H_2O$)	0·1
Potassium chloride	0·5
Ferrous sulphate ($FeSO_4.7H_2O$)	0·01
Water to	1000 ml.

The final media contained an excess of calcium carbonate, usually 12 g., as neutralizing agent. The salts were dissolved in water, the reaction adjusted to pH 7·6 and the solution diluted to 200 ml. The sucrose was dissolved in 800 ml. water. The salts and sucrose solutions were separately sterilized by autoclaving. The calcium carbonate was sterilized by dry heat. The three components of the medium were mixed aseptically immediately before inoculation; the reaction at this stage was usually pH 6·5–7·0.

General bacteriological methods

Stock strains and subcultures of *A. aerogenes* were stored on nutrient agar slopes. They were subcultured weekly, incubated for 24–48 hr. at 30° and kept until required at 2–5°. To prepare inocula for fermentations the organism was obtained in an actively dividing condition by transfer of a loopful of culture to 50–100 ml. of a liquid medium which was incubated at 35° for 16–18 hr.; one vol. of this culture was then transferred to five vol. of medium and a vol. of this dilution used such that the total volume of diluted inoculum amounted to 3 % of the total volume of fermentation mash. The inoculum culture was incubated at 35° for 7–8 hr. before addition to the main fermentation.

The following strains of *A. aerogenes* were used: B.199 (Northern Regional Research Laboratory of U.S. Department of Agriculture, Peoria, N.R.R.L. B. 199); 38 (N.C.T.C. No. 418); 39 (Dr G. A. Ledingham, National Research Council, Ottawa No. 5); 44 (Dr M. J. Johnson, University of Wisconsin, College of Agriculture, M 5 A 1).

Methods of analysis and expression of the fermentation results

The course of fermentation was followed by determination of the rate of decrease of sugar content and of production of 2:3-butylene glycol. The methods of analysis are described by Freeman & Morrison (1946). In addition to the rate of removal of sugar and of production of the glycol, completeness of sugar fermentation and yield of the product in terms of sugar initially present and of sugar fermented have also been used as criteria. In the tables the sugar contents have been expressed as g./l. of invert sugar. At the completion of incubation the sugar fermented (i.e. the difference between the initial and final invert sugar concentrations) was

expressed as a percentage of the initial invert sugar concentration under the heading of 'conversion %'. The 'yield % of sugar fermented' was obtained by expressing the final concentration of 2:3-butylene glycol as a percentage of the concentration of invert sugar fermented and similarly the 'yield % of total sugar' was obtained from the initial invert sugar concentration. Assuming that the fermentation may be expressed by the equation:

$$C_6H_{12}O_6 \rightarrow C_4H_{10}O_2 + 2CO_2 + H_2,$$

the theoretical yield of 2:3-butylene glycol is 50 % of the invert sugar fermented.

Fermentation in stirred and quiescent media

In the initial experiments, fermentation was carried out in unstirred media. This proved unsatisfactory owing to the slow establishment of equilibrium between calcium carbonate and the fermenting liquor; the reaction of the medium rapidly fell to sub-optimal values and fermentation was slow and incomplete. Under these conditions the volume of fermenting liquor had a pronounced effect on the reaction of the medium. Rapid establishment of equilibrium between the mash and calcium carbonate, and control of the reaction was obtained by mechanical stirring or rocking.

In the latter case 100 ml. of medium in a 350 ml. conical flask was suspended in a water bath thermostat and mechanically rocked at a rate of 100–105 cycles per min. with an amplitude of 6·4 cm. For this purpose a minor modification of the apparatus used for Warburg manometric studies proved satisfactory.

Table 1 gives the results of fermentations in quiescent, aerated and stirred mashes and under reduced pressure. When the fermentation was conducted under optimal conditions (Table 1 *a* and *b*), a rapid reaction was obtained with complete utilization of sucrose (initially 10 %) in 24–40 hr.

The course of the reaction fell into three characteristic phases:

(1) an initial lag phase of 5–7 hr. during which the maximum bacterial population was established. Under certain conditions, such as high initial carbohydrate concentration or in the presence of inhibiting substances, the lag phase may be 18–20 hr.;

(2) a period of 17–20 hr. of rapid sucrose utilization and glycol production during which these reactions followed a practically linear course. This period was characterized by the presence in the system of a virtually constant effective bacterial enzyme concentration and a large excess of substrate, with the result that reduction of substrate concentration and increase of product followed a linear course;

(3) when the substrate concentration fell below 0·5–1·0 % the rate of reaction decreased and there was a period of slow fermentation until completion.

Table 1. *Sucrose conversion and 2:3-butylene glycol production under various conditions*

(Organism: *Aerobacter aerogenes*, strain B. 199. Temperature of fermentation: 35°.)

Culture No.	Conditions	Invert sugar: g./l. at (hr.) 0	16	24	40	55	144	Final sugar conversion (% of initial sugar)	2:3-Butylene glycol + acetoin: g./l. at (hr.) 16	24	31	40	55	144	2:3-Butylene glycol yield % sugar fermented	% total sugar	pH data Time (hr.)	pH
(*a*)	100 ml. culture agitated. (F. 22)	101·0	43·0	8·9	3·7	—	—	97·0	20·2	34·6	—	37·4	—	—	37·6	37·1	40	6·9
(*b*)	2·5 l. culture stirred mechanically, aerated. (F. 27)	111·0	97·1 (8 hr.)	12·2	—	—	—	89·0 (24 hr.)	4·1 (8 hr.)	37·3	—	—	43·4 (72 hr.)	—	39·1	39·1	8 24 27	5·8 5·9 5·9
(*c*)	550 ml. culture unstirred, aerated at 25 l./hr. (F. 5)	102·9	—	54·4	47·9 (31 hr.)	36·8	23·5	79·4	—	10·1	16·8	—	16·7	27·5	34·6	26·7	144	5·4
(*d*)	As (*c*) but aerated at 50 l./hr.	102·9	—	45·1	37·8 (31 hr.)	32·2	6·5	96·4	—	13·5	24·2	—	—	31·0	32·2	30·1	144	5·9
(*e*)	550 ml. culture unstirred and unaerated	102·9	—	73·1	71·8 (31 hr.)	48·2	33·0	69·9	—	13·8	11·9	—	18·5	25·5	36·5	24·8	144	5·7
(*f*)	As (*e*) but absolute pressure 200 mm. Hg	102·9	—	70·7	58·8 (31 hr.)	47·1	37·5	65·4	—	12·6	14·6	—	18·8	23·2	35·5	22·6	144	5·6
(*g*)	2·5 l. culture unstirred and unaerated. (F. 14)	102·3	—	83·9 (20 hr.)	77·5 (44 hr.)	—	75·2 (91 hr.)	26·5	—	4·1 (20 hr.)	—	5·1 (44 hr.)	—	5·4 (91 hr.)	19·9	5·2	44 90	4·7 4·6

Investigation of variables affecting rate of fermentation and 2:3-butylene glycol production

The variables which affect the rate of fermentation and glycol yield were systematically investigated, including composition of the medium, concentration of substrate, pH, temperature and aeration.

Inoculum

The effect of variation of the volume of inoculum added to the main fermentation mixture was investigated over the range of 0·6–12 % (v/v). There was little variation in the rate of sugar conversion and 2:3-butylene glycol yield throughout the range of inoculum concentration from 0·6–12 % (v/v). There was, however, some indication that highest glycol yields (though slightly slower fermentation) were obtained with smaller inocula. This may be due to the production of a greater number of generations in the fermentation medium giving a better acclimatized organism and a more vigorous culture. An inoculum level of 3 % was chosen for subsequent work but the results showed that this might be decreased to 0·6 % or even lower without appreciable diminution in the rate of fermentation. The use, for the inoculum, of either a synthetic medium based on the fermentation medium described, a glucose + salts + corn steep liquor medium, or a beer wort + calcium carbonate medium, had no significant effect on the rate of fermentation. The use of a synthetic medium for this purpose is recommended.

Organic nutrients such as yeast extract

Although *A. aerogenes* is apparently able to synthesize all its necessary growth factors, the effect of addition of such factors on the rate of fermentation was investigated. The effect of addition of yeast extract clearly showed that such additional organic nutrients played no part in the fermentation process and were unnecessary. Similar negative results were obtained with yeast autolysate and malt rootlet extract.

Optimal concentration of salts

Ammonium sulphate. In the early work on *A. aerogenes* fermentations peptone was used as the nitrogenous nutrient (Harden & Walpole, 1906). More recent work has shown that amino-acids (Lodge & Hinshelwood, 1944), urea and ammonium salts (Verhave, 1928) are satisfactory for this purpose. In the present work, ammonium sulphate was used as the sole source of nitrogen. In preliminary experiments to determine the optimal concentration an excess of the salt was added and by difference the amount of ammonia assimilated by the bacteria at the completion of fermentation was determined. This was found to be about 2·6 g./l. expressed as ammonium sulphate. The effect of a series of ammonium sulphate concentrations between zero and 10 g./l. on rate of fermentation and glycol yield was determined. The rate of fermentation and glycol yield were practically independent of ammonium sulphate concentration in the range 4–10 g./l. In subsequent experiments an initial ammonium sulphate concentration of 4 g./l. was used.

Dipotassium hydrogen phosphate. By similar means to that described above the optimal K_2HPO_4 concentration was found to be 2·5 g./l.; increase of phosphate concentration to 10 g./l. did not influence the characteristics of fermentation. In the absence of added phosphate (other than the traces introduced with the inoculum) fermentation was greatly suppressed; only 40 % conversion of sucrose took place in 24 hr. and the glycol yield was only about 30 % of the maximum possible.

Determination of optimal pH

The products of the *A. aerogenes* fermentation of hexoses include 2:3-butylene glycol, acetoin (acetylmethylcarbinol), ethanol, lactic acid and formic acid (Ledingham & Adams, 1944). Lactic acid is the main acidic product; as described below it has been obtained to the extent of 5–10 % of the hexose fermented. It is necessary to neutralize this lactic acid as it is formed in order to maintain an optimal pH. This may be done either by continuous addition of soluble alkali or by maintaining an excess of calcium carbonate in equilibrium with the fermenting liquor. The effect of pH on rate of fermentation and glycol production was investigated in three ways; by variation in the amount of calcium carbonate suspension, by maintenance of a constant pH by addition of soluble alkali and by a washed suspension technique in which no bacterial growth took place.

Variation of amount of calcium carbonate suspension. A series of fermentations was carried out in agitated 100 ml. mashes in which the calcium carbonate contents were varied from 0–12 g./l. The pH of the fermenting mixtures was recorded at intervals (glass electrode). The results showed that complete conversion was obtained in 41 hr. or less with $CaCO_3$ from 3–12 g./l. The initial rates of sucrose conversion and 2:3-butylene glycol formation were greatest with the highest amounts of calcium carbonate but did not differ greatly between 9 and 12 g./l. The mean pH values during the period of vigorous fermentation were pH 5·78, 5·52, 5·40, 5·03 and 4·08 for calcium carbonate additions of 12, 9, 6, 3 and 0 g./l. respectively, and the corresponding yields of 2:3-butylene glycol in terms of sugar fermented were 35·8, 36·5, 39·3, 41·3 and 41·0 % respectively. Whilst fermentation was most rapid in the pH range 5·4–6·0 the glycol yield was at a maximum at pH 5·03–5·4.

Washed suspension technique. Preliminary experiments with washed suspensions of 17 hr. cultures of *A. aerogenes* B. 199 showed that on addition of 0·5–2·0 % solutions of glucose at 35°, the latter was fermented with the production of 2:3-butylene glycol as the main non-gaseous metabolic product. When the bacterial concentration was less than 1·30 g. dry weight/l. in 0·5 % glucose, concentration of enzyme system was the limiting factor controlling rate of fermentation and the rate of reaction was a linear function of time until the bulk of the glucose had been converted. The rate of fermentation, under these conditions, was proportional to the concentration of bacterial suspension.

The organism was grown on either a glucose + peptone + Lemco medium or on a glucose-containing synthetic medium buffered at pH 6·8 with 0·05 M-phosphate + citrate buffer at 35° for 17–20 hr. During growth the reaction fell to pH 5·5. The bacterial cells were separated by centrifuging, washed with buffer (pH 6·8) and resuspended in buffer to give a washed suspension containing the equivalent of 10·9 g. dried cells/l. This was used to prepare the suspensions used to obtain the results recorded in Table 2.

The buffering power of the medium was insufficient to overcome the acidity of the lactic acid produced and a marked fall of pH took place particularly in the early stages of the reaction. The quantity of glucose fermented was greatest at a mean pH of 5·57 and fell progressively with the more acid media. The yield of 2:3-butylene glycol in terms of glucose fermented was greatest at a mean pH of 4·8 (86·6 % of theory) decreasing to 61·2–71·0 % of theory at pH 5·50–5·57.

Control of pH *by addition of soluble alkali.* Control of pH during fermentation by stepwise addition of soluble alkali (sodium hydroxide or ammonia) had the great advantage over the use of an excess of calcium carbonate that there was more accurate control of reaction at any chosen point in the range pH 4·5–8.

Two fermentations of sucrose (2·5 l. scale) were conducted in which the pH was maintained at (*a*) 6·0–6·5, and (*b*) 5·5, by stepwise addition of 2·5 N-NaOH. The solutions were aerated at a rate of 100 l. air/hr.; incubation temperature, 35°; organism, *A. aerogenes* B. 199. A calomel-glass electrode cell was built into the fermentation vessel and connected directly to the pH meter so that determinations could readily be made without the necessity of sampling. The fermenting liquor was gently stirred to maintain the bacterial growth in suspension and to avoid high local alkalinity on addition of the NaOH. The pH was determined at 15–30 min. intervals during the initial 32 hr. of fermentation and the reaction restored to pH 6·5 or 5·5, respectively, by addition of a suitable volume of the alkali. Determinations of sucrose and 2:3-butylene glycol were made at 3 hr. intervals.

Table 2. *Determination of optimal* pH *by washed suspension technique*

(The following mixtures were prepared: 10 ml. bacterial suspension (concentration: 10·9 g. dried cells/l.) + 5 ml. 20 % glucose solution + 35 ml. 0·5 M-phosphate buffer at the following pH: (1) 5·97; (2) 6·47; (3) 7·00; (4) 7·50. Initial glucose concentration: 18·31 g./l. Incubation at 35°.)

Initial pH	Mean pH during fermentation	Glucose after 22 hr. (g./l.)	Glucose fermented (by difference) (g./l.)	Yield of 2:3-butylene glycol in 22 hr. (g./l.)	Yield as % of sugar fermented
5·97	4·86	8·32	9·99	4·32	43·3
6·47	5·14	3·93	14·38	5·70	39·6
7·00	5·50	2·78	15·53	4·75	30·6
7·50	5·57	1·53	16·78	5·95	35·5

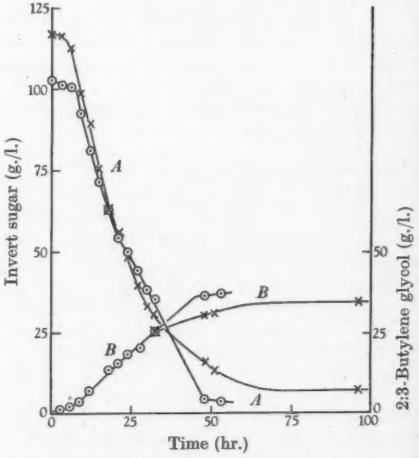

Fig. 1. pH control by stepwise addition of sodium hydroxide. Fermentations of sucrose by *A. aerogenes* B. 199 (2·5 l. scale) were carried out in which the pH was maintained at (I) 6·0–6·5 and (II) 5·5, by stepwise addition of 2·5 N-sodium hydroxide at 15–30 min. intervals. Incubation temperature, 35°; aeration, 100 l. air/hr. ×—× pH 6·0–6·5; ⊙—⊙ pH 5·5. *A*, invert sugar fermentation; *B*, 2:3-butylene glycol production.

The results (Fig. 1) confirmed the existence of a lag phase of fermentation of about 6 hr. duration. The rates of fermentation and 2:3-butylene glycol production were slower than in similar fermenta-

tions in the presence of calcium carbonate (cf. F. 27 (*b*), Table 1), but this was probably due to the unavoidable width of the pH steps between successive alkali additions rather than to any stimulating effect of calcium ions. At pH 6·0–6·5 the initial rate of fermentation (sucrose conversion and glycol production) was 25–30% greater than at pH 5·5, but the 2:3-butylene glycol yield was 22% higher at the lower pH value. Lactic acid formation was favoured by increase of pH. Thus in the experiment at pH 6·0–6·5 the alkali required for neutralization was equivalent to 18·95% formation of lactic acid in terms of sugar fermented, while in the experiment at pH 5·5 the corresponding yield of lactic acid was only 10·62%. The sum of 2:3-butylene glycol and lactic acid yields in these experiments, viz. 50·7 and 49·3% was approximately constant. The fermented liquors were very opalescent owing to the bacterial growth, part of which flocculated.

Effect of initial carbohydrate concentration

The influence of the initial sucrose concentration on the rate of sugar conversion and 2:3-butylene glycol yield was investigated in the range of 5–20% sucrose concentration.

In each case 100 ml. of medium was agitated in a 350 ml. flask at 35°. The amounts of calcium carbonate added were kept constant at 12 g./l. Under conditions of free aeration provided by means of a cotton-wool plug in the flask, acetoin began to replace 2:3-butylene glycol as the product of the reaction after the sugar concentration had fallen to less than 1%. To minimize acetoin formation the cotton-wool plug was replaced by a rubber stopper connected by a short length of glass tubing to a Bunsen valve when the carbohydrate concentration had fallen to about 1%. By this means free evolution of carbon dioxide from the flask was possible but ingress of air was greatly impeded. There was an increase in the duration of the initial lag phase of sugar fermentation with increasing carbohydrate concentration. With initial sucrose concentrations of 5 and 10% the lag periods were 5–6 hr. At 15 and 20% initial sucrose concentrations they had increased to 9–10 hr. and 15–16 hr. respectively. The yield of 2:3-butylene glycol was at a maximum (87·4% of theory) with an initial sugar concentration of 15%. The slope of the curves in the rapid, linear phase of fermentation (Fig. 2) increased slightly with increasing initial sugar concentration, but owing to the longer lag phase and greater duration of the linear period the overall time of fermentation increased with increasing initial sugar concentration. Fermentation was complete in 20, 25, 35 and *c.* 47 hr. with initial sucrose concentrations of 5, 10, 15 and 20% respectively.

According to Fulmer, Christensen & Kendall (1933) the optimal initial sucrose concentration is 8%, but under the conditions used by these workers fermentation was very slow, requiring 4 days and 23 days for completion at initial sucrose concentrations of 1 and 10% respectively.

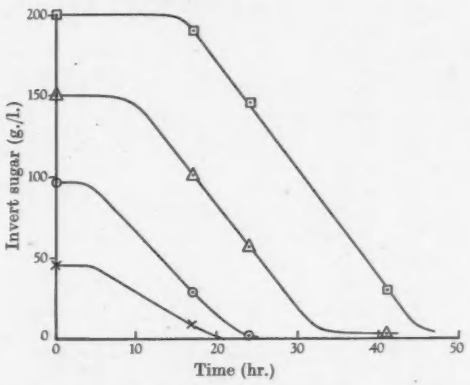

Fig. 2. Effect of initial carbohydrate concentration. 100 ml. quantities of medium containing a series of different initial sucrose concentrations in the range of 5–20% were fermented by *A. aerogenes* B. 199 at 35°. Aeration was restricted after the sugar concentration had fallen below 1%. A constant quantity (equivalent to 12 g./l.) of $CaCO_3$ was added to each fermentation. Invert sugar concentration initially: 45·6 g./l. ×—×; 97·2 g./l. ⊙—⊙; 149·7 g./l. △—△; 200·4 g./l. ⊡—⊡.

Determination of optimal temperature

The rates of sucrose fermentation and 2:3-butylene glycol production in *A. aerogenes* B. 199 fermentations were determined at intervals in the temperature range 25–40°. The fermentations were conducted with 100 ml. of medium in 350 ml. flasks with agitation. The temperature was maintained by immersing the flasks in a thermostatically controlled water bath, with a variation of less than ± 0·1° of the stated temperatures. In series I (Table 3) the fermentation vessels were closed only with cotton-wool plugs which allowed free aeration; in series II free aeration was allowed in the initial stages of fermentation during which a high concentration of sugar was present, and after 17 hr. the plugs were replaced by Bunsen valves as described above. The results (Table 3) show that:

(*a*) Practically complete sugar conversion was obtained under aerobic conditions with temperatures in the range 30–37·5° in a 41 hr. fermentation. Outside this temperature range fermentation was much slower and was not complete in 41 hr. With restricted aeration much lower conversions were obtained in the same period, but maximum conversion was again obtained at 35° (89·2%).

Table 3. *Determination of optimal temperature*

(100 ml. volumes of medium inoculated with *A. aerogenes* B. 199 were agitated in 350 ml. flasks, in thermostatically controlled baths at a series of temperatures in the range 25–40°.)

Exp. no.	Temperature	Initial invert sugar (g./l.)	Conversion after 41 hr. (%)	Initial rate of sugar conversion (g./l./24 hr.)	Initial rate of glycol formation (g./l./24 hr.)	Final 2:3-butylene glycol (g./l.)	Glycol yield (% sugar fermented)	pH at 41 hr.
			I. With cultures freely aerated					
46 I	25·0	110·9	68·2	47·1	14·0	23·8	31·4	6·04
46 A	30·0	116·3	96·6	66·3	18·3	37·8	33·7	6·53
46 E	30·0	109·1	93·5	65·7	20·2	36·7	36·0	5·86
46 G	32·5	115·4	93·3	73·5	20·2	36·0	33·4	6·27
41	35·0	111·5	100	105·6	35·6	39·5	35·8	6·91
46 H	37·5	112·1	87·5	57·6	16·4	31·9	32·5	5·69
46 F	40·0*	110·1	73·8	25·5	5·2	25·6	31·5	5·40
			II. With restricted aeration					
46 I	25·0	110·9	62·3	47·4	12·6	19·5	28·2	5·82
46 A	30·0	116·3	68·4	58·1	14·3	22·9	28·8	6·20
46 G	32·5	115·4	80·1	66·9	17·8	27·4	29·7	6·07
41	35·0	111·5	89·2	87·4	28·0	32·6	32·8	5·54
46 H	37·5	112·1	47·6	41·3	12·3	15·7	29·5	5·73

* Lag phase lasted about 17 hr.

(*b*) The initial rates of sucrose conversion and 2:3-butylene glycol production, both under aerobic and 'anaerobic' conditions showed sharp maxima at 35°. There was a very steep fall with increasing temperature and a slower fall of these rates towards lower temperatures. The optimal temperature was 35·0°. According to *Bergey's Manual* (1940) the optimal temperature for *growth* of this organism is about 30°.

(*c*) Variation in temperature had comparatively little effect on yield of 2:3-butylene glycol in terms of sucrose fermented but there was a definite maximum at 35°.

Effect of different strains of Aerobacter aerogenes

Within the species *A. aerogenes* there exists a large number of different strains which, though morphologically identical, differ in their rates of fermentation and yields of metabolic products. Reversible and irreversible changes in a given strain may also be induced by acclimatization to new growth media. Lodge & Hinshelwood (1944) showed how the organism could adapt itself to certain changes in source of carbohydrate and nitrogenous nutrients and Perlman (1944) obtained higher yields of 2:3-butylene glycol and faster fermentation of wood hydrolysates with acclimatized strains than with the parent organism.

A number of strains obtained from other workers have been compared in fermentations of sucrose at 35°. A few strains have also been isolated from natural sources such as grains, plants, faecal matter and soil; those whose production of acetoin was demonstrated by the Voges-Proskauer reaction were submitted to fermentation tests to determine production of 2:3-butylene glycol. So far only a number of disappointingly slow fermenters have been obtained which gave only 23–25% of 2:3-butylene glycol in terms of sugar fermented. Strains N.C.T.C. 418, M 5 A 1/L and S were fast fermenters with which practically complete conversion of sucrose was obtained in 40·5 hr. with high glycol yields. They were, however, all inferior to the B. 199 strain. The Wisconsin strain M 5 A 1 as received was separated into two forms M 5 A 1/L and S, characterized by large and small colonies on nutrient agar, respectively. There was an appreciable difference of rate of fermentation between these strains, the former being the more rapid fermenter.

Effect of aeration on fermentation

Kluyver, Donker & Hooft (1925) first showed that the presence of hydrogen acceptors, including oxygen, stimulated the production of acetoin and 2:3-butylene glycol in yeast fermentations; this principle was applied to *A. aerogenes* fermentations by Verhave (1928). According to Scheffer (1936) the accelerating effect of aeration on 2:3-butylene

glycol production is not due to a specific physiological action of oxygen but to a diminution in the concentration of carbon dioxide and he claimed that the use of other gases such as hydrogen or nitrogen or a reduction of pressure had a similar effect.

Our experiments have confirmed the accelerating effect of aeration and shown that higher yields of 2:3-butylene glycol in terms of sugar fermented are obtained in aerated than in unaerated cultures. This effect was evident in the series of experiments recorded in Table 3, but the full effect of aeration was somewhat masked as in series II the aeration was restricted only after the first 17 hr. of fermentation.

In order to determine the effect of unaerated conditions throughout the period of fermentation a stirred sucrose-containing medium (2·5 l.) was fermented at 35°, using strain B. 199. Egress of carbon dioxide from the flask was allowed by means of a glass tube (5 mm. bore) tightly closed with a cotton-wool plug. A similar culture was aerated at 150 l. air/hr. (Table 4). From interpolated values in exp. 38 (in parentheses in Table 4) the rates of sucrose attenuation and 2:3-butylene glycol production during the linear phase of the reaction under aerobic conditions (exp. 27) were 1·6 and 2·2 times respectively, the corresponding rates under unaerated conditions. Under both sets of conditions complete fermentation was obtained; in the aerated culture the reaction was shown graphically to be complete in 30–32 hr. and in the unaerated culture complete conversion of sugar was not reached until 91 hr., although 95·9 % of the initial sugar had been fermented in 51 hr. and 98·9 % in 67 hr. The yield of 2:3-butylene glycol was 32 % higher under aerated conditions than under unaerated; in the former it reached 39·1 % (i.e. 78·2 % of theory) and in the latter 29·6 % (59·2 % of theory).

Table 4. *Effect of aeration on fermentation*

(Cultures of *A. aerogenes* B. 199 were allowed to ferment at 35° with and without aeration.)

	Changes during 'anaerobic' fermentation 2·5 l. scale. Exp. 38		Changes during fermentation of 2·5 l. culture with aeration (150 l. of air/hr.). Exp. 27	
Time (hr.)	Invert sugar (g./l.)	2:3-Butylene glycol (g./l.)	Invert sugar (g./l.)	2:3-Butylene glycol (g./l.)
0	115·0	—	111·0	—
3·5	110·0	2·2	—	—
8	—	—	97·1	4·05
19	76·5	10·7	—	—
23	55·8	15·5	—	—
24	(53·2)	(16·7)	12·2	37·3
27	45·3	20·4	—	*
43	12·6	28·5	—	—
51	4·7	30·8	—	—
67	1·2	33·2	—	—
72	0·44	33·8	0·0	43·4
91	0·0	34·0	—	—
116	0·0	34·0	—	—
2:3-Butylene glycol as % of total sugar	29·6		39·1	
2:3-Butylene glycol as % of theoretical	59·2		78·2	

* Aeration and stirring arrested.

Products of the Aerobacter aerogenes *fermentation*

Characterization of the d- *and* meso-2:3-*butylene glycol mixture*

In order to establish the nature of the glycol produced in this work, samples were isolated as described by Freeman & Morrison (1947) and their properties determined. The pure product after fractional distillation *in vacuo* had the properties recorded in Table 7 which agree satisfactorily with the literature data for *d*- and *meso*-mixtures of the glycol.

The differences of m.p., b.p. and refractive index exhibited by the three preparations of 2:3-butylene glycol are due to the different proportions of the *d*- and *meso*-isomers present in the mixtures. The product obtained in the present work appears to contain more of the *d*-isomer than the glycols isolated by Harden & Walpole (1906) or Morell & Auerheimer (1944). Its content of the *d*-isomer is calculated from the optical rotation data to be approximately 14 %.

2:3-Butylene glycol diphenylurethane and dibenzoate were prepared by treatment with phenylisocyanate and benzoyl chloride, respectively.

The diphenylurethane was obtained as a colourless crystalline solid, m.p. 197° (corr.) (authentic value 199°) and gave on analysis C, 65·89, 65·60; H, 5·78, 6·16; N, 8·47, 8·54. $C_{18}H_{20}O_4N_2$ requires C, 65·85; H, 6·16; N, 8·54%. The dibenzoate was a colourless crystalline solid, m.p. 76–77° (literature value, 77°). The dibenzoate gave an equivalent on hydrolysis with ethanolic KOH of 145 (theory 149).

During the crystallization of the pure diphenylurethane, traces of other urethanes were obtained but all attempts to isolate an isomeric diphenylurethane melting at 151° as reported by Walpole (1911) were unsuccessful. Similarly, only a single dibenzoate was isolated. It was concluded that the main non-gaseous product of *A. aerogenes* fermentation of sucrose was a *d*- and *meso*-mixture of 2:3-butylene glycol isomers in which the *meso*-compound greatly predominated.

Acetoin

The formation of 2:3-butylene glycol in bacterial fermentation appears to take place through the intermediate stages of pyruvic acid, acetaldehyde, acetoin and finally reduction of the latter to 2:3-butylene glycol. Under aerobic conditions, the glycol is oxidized by *A. aerogenes* yielding acetoin, or diacetyl if the aeration is excessive (Stanier & Fratkin, 1944). Our results have confirmed the production of acetoin and shown that even under aerobic conditions appreciable oxidation of 2:3-butylene glycol to acetoin does not take place until the sucrose concentration has decreased to 1% or less. In experiments under aerobic conditions (particularly those in which the cultures were agitated in cotton-wool plugged vessels) appreciable quantities of acetoin were produced when the sugar conversion was allowed to proceed to completion. In these cases acetoin was determined by the method of Langlykke & Peterson (1937) and suitable corrections were made to the reducing sugar and 2:3-butylene glycol values as described by Freeman & Morrison (1946).

Acetoin production in fermentations of sucrose under different conditions of aeration are summarized in Table 5. Under anaerobic conditions (exp. 38) no acetoin was present after 116 hr. fermentation, despite the fact that over 95% of the sugars were fermented in the first 51 hr. When 100 ml. of culture were freely aerated throughout the fermentation period of 40·5 hr., acetoin production reached 27 g./l., i.e. 24·2% of hexose fermented (exps. 41/4 and 5). When the aeration was partly restricted after 16 hr. (43·8% unfermented sucrose

Table 5. *Acetoin production by* Aerobacter aerogenes *B.* 199 *in aerated cultures fermented under various conditions*

Conditions	Time of fermentation (hr.)	Exp. no.	Acetoin (g./l.)	Unfermented sugar as invert sugar (g./l.)	2:3-Butylene glycol (g./l.)	Acetoin (% of sugar fermented)	2:3-Butylene glycol (% of sugar fermented)
Anaerobic 2·5 l. culture	116	38	Nil	Nil	34·0	Nil	29·6
100 ml. culture, aeration partly restricted by Bunsen valve plug after 16 hr. when 44% unfermented sugar present	40·5	41/1	3·35	12·1	30·6	3·4	30·8
		41/2	3·80	0·5	32·3	3·42	29·1
		41/3	2·96	9·7	29·7	2·91	29·2
	(Mean of above)		3·37	7·4	30·9	3·2	29·7
As above but freely aerated for total 40·5 hr. period	40·5	41/4	27·0	0·0	12·1	24·2	10·9
		41/5	27·0	0·0	13·1	24·2	11·7
	(Mean of above)		27·0	0·0	12·6	24·2	11·3
Fermentation of glucose, 12·5 l. culture. Aeration 100 l./hr. until 46 hr. when 0·89% sugar unfermented	91	44	0·9	6·8 (as glucose)	29·2	0·84	27·1
Varying initial sucrose concentration. 100 ml. cultures, aeration partly restricted when following amounts of unfermented invert sugar were present (as g./l.):							
1. 8·9 (17 hr.)	65	45/1	4·5	Nil (45·6)	10·7	9·9	23·4
2. 2·4 (24 hr.)	65	45/2	9·7	Nil (97·2)	30·3	10·0	31·1
3. 3·6 (41 hr.)	65	45/3	9·5	Nil (149·7)	55·9	6·3	37·4
4. 3·0 (49 hr.)	65	45/4	4·7	3·6 (200·4)	77·4	2·4	39·3

Initial sugar concentration in parentheses

as invert sugar present) the corresponding acetoin yields were only 3·4 g./l. (3·2 % of sugar fermented). Similar evidence of acetoin production under aerobic conditions when the sucrose concentration had fallen to 1 % or less is presented in Table 6 (exps. 44 and 45). Acetoin production takes place during the third, slow phase of fermentation described above.

1945) 2:3-butylene glycol (85 % of theory), acetoin (3 % of theory) and ethanol (4 % of the theoretical based on glucose fermented). Similar results have been obtained in the present work (Table 6) but under certain conditions, particularly with unaerated cultures, high yields of ethanol (12–14 % of the sugar fermented) and of lactic acid (5–10 %) were obtained.

Table 6. *Yields of non-volatile metabolic products of fermentation of sugars by* Aerobacter aerogenes *under various conditions (as % of sugar fermented)*

	Conditions of fermentation			
Products	Unaerated fermentation of sucrose 2·5 l. culture 91 hr. (Exp. 38)	Glucose fermentation with aeration at 100 l./hr. 12·5 l. culture 91 hr. (Exp. 44)	Sucrose fermentation with aeration 100 ml. culture. Initial concentration of sucrose 149·7 g./l. (Exp. 45/3)	As 45/3 but initial sucrose concentration 200·4 g./l. (Exp. 45/4)
2:3-Butylene glycol	29·6	27·1	37·4	39·3
Acetoin	Nil	0·8	6·3	2·4
Lactic acid	5·6	9·8	—	—
Ethanol	13·6	12·1	—	—
Total non-gaseous products	48·8	49·8	—	—

Table 7. *Properties of* d- *and* meso-*mixtures of* 2:3-*butylene glycol isomers isolated from* Aerobacter aerogenes *fermentations*

	Product from *A. aerogenes* B. 199 fermentations	*d*- and *meso*-mixtures of 2:3-butylene glycol isomers	
		(*a*) Harden & Walpole (1906)	(*b*) Morell & Auerheimer (1944)
m.p.	11–12°	14°	25°
b.p.	183·0°	180°	180–182°
Refractive index:			
$n_D^{21·5}$	1·4371	1·4360 (27°)	1·4381 (18°)
$[\alpha]_{5461}^{20°}$	+2·27	—	—
$[\alpha]_D^{23°}$	+1·80	+0·82 (25°)	+1·06
Density 20°/4°	1·002	—	1·000 (25°)

Other products

The main non-gaseous products of the *A. aerogenes* fermentation are 2:3-butylene glycol, acetoin, ethanol, formic acid and lactic acid (Adams & Stanier, 1945). Acid-hydrolyzed starch mashes fermented by *A. aerogenes* yielded (Ward *et al.*

SUMMARY

1. The following factors affecting the conversion of sucrose to 2:3-butylene glycol by *Aerobacter aerogenes* have been investigated: pH, sucrose concentration, optimal temperature, aeration, and addition of organic nutrients.

2. The pH optimum for production of the glycol was 5·0–5·5 and the optimal temperature, 35°.

3. Rapid fermentation and high 2:3-butylene glycol yields were obtained with initial sucrose concentrations in the range 5–20 %. Maximum yield of the product (87·4 % of theory) was obtained with an initial sucrose concentration of 15 %.

4. In aerated stirred cultures containing 10 % sucrose and 1·2 % calcium carbonate as neutralizing agent, rapid fermentation began after a lag phase of 5–7 hr. and was complete in *c.* 24 hr.

5. The main non-gaseous products were *d*- and *meso*-2:3-butylene glycol, ethanol, and lactic acid. Acetoin was not formed under anaerobic conditions, but replaced the glycol as the major product when aeration was continued after the carbohydrate concentration had fallen to 1 % or less.

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The Turnover of Phosphate in the Pineal Body Compared with that in Other Parts of the Brain

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The metabolism of the brain has been investigated by various methods. Chute & Smyth (1939) and Broman & Jacobsohn (1941) have shown that the oxygen consumption of isolated perfused cat brain amounts to about 100–300 ml. O_2/100 g. of brain/hr. An approximate calculation of the Q_{O_2} results in values of between 4 and 12 ($Q_{O_2} = \mu$l./hr./mg. dry weight.) Another method of determining the metabolism of the brain consists in measuring, in the intact brain *in situ*, the composition of the arterial blood flowing into and the venous blood flowing from the brain. This method is however less suitable for obtaining absolute values, because the velocity of the blood streaming through the brain must be evaluated. This meets with certain difficulties.

In the method most usual at present, metabolism is measured in surviving brain slices by the Warburg procedure. The Q_{O_2} values thus found for cortex slices have usually varied between 5 and 10. This method also shows that the metabolism of the brain is chiefly associated with carbohydrates. Pyruvic acid is one of the most important metabolic products. The oxidation of this substance in the brain has been studied by, for example, Simola & Alapenso (1943) and Long (1943). A number of enzymic reactions takes place in the brain, but no scheme for the carbohydrate metabolism in this tissue has yet been established (Evans, 1944).

Transphosphorylation in the brain has also been studied by this method. Macfarlane & Weil-Malherbe (1941) thought that the breakdown of glucose in the brain takes place with the aid of a cycle of phosphate transfers. This supposition was based on the observation that inorganic phosphate in slices of rat brain undergoes no change during anaerobic glycolysis, whereas the pyrophosphate is reduced to one-third of its original value. Borell & Örström (1945) showed later that radioactive phosphate chiefly accumulates as hydrolyzable acid-soluble phosphate esters, which supports the above supposition.

The employment of P^{32} constitutes an advance in technique. This method allows us to measure, in very small parts of the brain, the velocity at which the marked P atom participates in the metabolism. The phosphorus participates, however, not only in the carbohydrate metabolism, but also in that of the phospholipids and nucleic acids. These latter processes in the brain have been studied by several workers (Chargaff, 1939; Chargaff, Olson & Partington, 1940; Hevesy & Hahn, 1940; Hevesy & Ottesen, 1943; Hevesy, 1940).

The investigations of Hevesy & Hahn (1940) show that a strikingly low metabolism is found in the brain, when measured by means of radioactive P; this we are in a position to confirm. Their subsequent work furnished further evidence for the view of Hevesy & Ottesen (1943) that there is only a low metabolism in the brain. These authors isolated deoxyribonucleic acid from the brain and the metabolism proved to be very low even in this quantitatively considerable fraction.

In our investigations we attempted to divide the brain into sections comparatively well-defined anatomically, and to compare their phosphate metabolism by means of radioactive P. A very unexpected result was obtained, in that the pineal body was found to be the most active portion of the brain (Borell & Örström, 1945). These problems will be dealt with further in this work.

MATERIAL AND METHODS

Rats were generally used in the present investigation. Each animal was injected intraperitoneally with 1 ml. of a solution of radioactive P (as sodium phosphate) containing 0·03–0·04 mc./ml. 5% glucose. The concentration employed gave an activity readily measurable in the small parts of the brain that were investigated.

The animals were decapitated, usually 40 min. after the injection. Blood (0·2 ml.) was drawn off immediately after decapitation, after which the portions of the brain were dissected according to the scheme of Borell & Örström (1945). Table 2 gives the parts of the brain investigated.

Table 1. *Calculation of relative specific activity*

Organ	Radio-activity (impulses/min.)	Phosphorus content (μg.)	Sp. activity	Rel. sp. activity
Pineal	137	71·5	1·92	33
Cerebellum	130	2280·0	0·058	1
Blood	800	149·0	5·35	92

The values are expressed in terms of relative specific activity. The way in which this is calculated is seen from Table 1. First the specific activity is calculated, this being the number of impulses/μg. P. To obtain values which are comparable with one another and not so highly dependent on the biological variations of the material, the specific activity in the different organs was referred to a standard. We have chosen the specific activity of cerebellum as such a standard and the values for all other parts of the brain in the same experiment have been referred to it. Values thus obtained are called relative specific activities.

Table 2. *Specific activity and relative specific activity in different parts of the brain of the rat*

(The animals were killed 40 min. after injection of P^{32}.)

Part	No. of animals	Sp. activity	Rel. sp. activity
Pineal body	36	8·2 ±1·8	26·5±5·5
Pituitary: ant. lobe	36	2·14±0·42	8·6 ±1·2
Pituitary: post. lobe	30	2·61±0·53	7·9 ±1·4
Choroid plexus	24	3·27±0·71	11·2 ±2·8
Olfactory lobe	18	0·41±0·08	1·30±0·17
Grey matter of brain	9	1·05±0·17	1·74±0·30
White matter of brain	9	1·19±0·54	1·98±2·50
Telencephalon	21	0·31±0·06	0·76±0·13
Tuber cin.: Ant. part	24	0·39±0·05	1·12±0·15
Post. part	24	0·48±0·11	1·20±0·14
Cent. part	21	0·62±0·12	2·72±0·46
Habenula	27	1·10±0·27	2·68±0·48
Thalamus	24	0·31±0·07	0·78±0·12
Mammillary body	24	0·58±0·14	1·49±0·95
Perforated substance	24	0·42±0·09	1·16±0·80
Corpora quadrigemina	24	0·27±0·01	0·69±0·07
Pons	24	0·28±0·02	0·72±0·17
Medulla oblongata	24	0·46±0·10	1·02±0·23
Cerebellum	36	0·33±0·05	1·00
Blood	36	19·9 ±4·35	40·5 ±6·6

RESULTS

As shown in Table 2 the different parts of the brain have been divided according to their activities into two distinct groups. The first group contains very active parts of the brain. It consists of the pineal, the anterior and posterior lobes of the pituitary and the choroid plexus. The other group comprises the fifteen other parts tested. Finally the relative specific activity of the blood also figures in the table (cf. also Borell & Örström, 1945).

The very powerful activity of the pineal seems to be the most remarkable phenomenon. The value of the relative specific activity exceeds that of the pituitary in all cases. The mean value is about 3–4 times the average value of the anterior and posterior lobes.

The values in Table 2 are for animals killed 40 min. after injection of P^{32}. For the purpose of determining the importance of the time elapsing between the injection and the killing of the animals, a series of experiments was carried out at different times after the injection (Figs. 1 and 2). The specific activities of all parts are high 40 min. after the injection. In parts of brain such as the pineal, choroid plexus and the anterior and posterior lobes of the pituitary body, on the other hand, the specific activity shows a strong diminution as early as about 2 hr. after the injection (Fig. 1). In parts with low metabolism, however, the decrease is insignificant (Fig. 2). No appreciable change from the two-hour values was observed 24 hr. after the injection.

The specific activity decreases most rapidly in the blood. This reduction is due to the fact that free phosphate disappears very rapidly (Table 3).

Table 3. *The turnover of different phosphate fractions in the pineal body and blood at different intervals after the injection of radioactive* P

Phosphate fraction	Pineal impulses No.	Pineal impulses %	Blood impulses No.	Blood impulses %
	40 min.			
Free phosphate	357	35	3450	90
Total organically bound P	667	65	398	10
10 min. hydrolysis	463	45	110	3
Residual fraction	167	17	240	6
Precipitable by trichloro-acetic acid	37	3	48	1
	2 hr. 10 min.			
Free phosphate	40	28	2320	43
Total organically bound P	103	72	3080	57
10 min. hydrolysis	14	10	1330	25
Residual fraction	62	43	1550	29
Precipitable by trichloro-acetic acid	27	19	198	3

The question arises as to whether the high phosphate turnover in the pineal is connected with a lively turnover of nucleic acid and phospholipids or with a vigorous metabolism of the phosphorylated intermediate compounds appearing

in the carbohydrate cycle, or whether it is just a diffusion of free phosphate from the blood.

By means of hydrolysis we have attempted to determine the percentages of phosphate in different fractions. The results are given in Table 3.

and 72% respectively after these two periods. If, however, the radioactivity is determined in the individual organic phosphate ester fractions, a distinct change is observed. The fraction obtained after 10 min. hydrolysis shows a strong diminution,

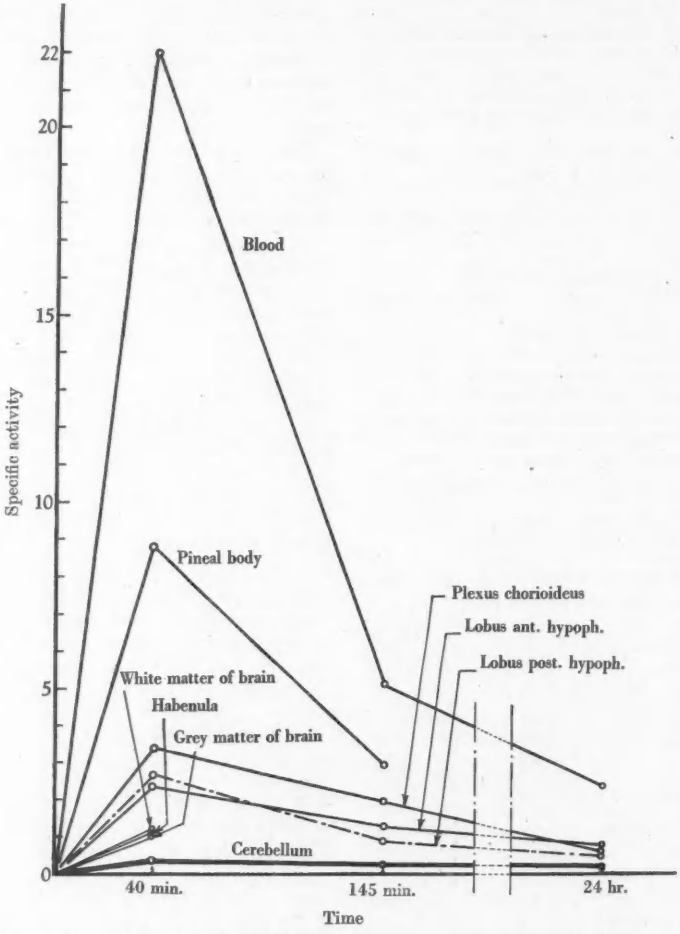

Fig. 1. The specific activity in different parts of the brain from rats at different times after the injection of radioactive phosphorus. In the figure are shown parts of the brain with a specific activity greater than 1·0, with the exception of cerebellum which was chosen as a basis for comparison.

This table gives only the numbers of impulses from the different P fractions and their percentage distribution. Blood has been included as a standard for comparison. The radioactivity in the inorganic phosphate fraction from the pineal is 35% after 40 min. and 28% after somewhat over 2 hr. The sum of the organic phosphate esters constitutes 65

while the residual fraction and that precipitable by trichloroacetic acid increase after 2 hr. 10 min. Each phosphate fraction is characterized by the rapidity with which it turns over radioactivity. The maximum in the curve appears first for the rapid intermediate links and later for the slow links (Örström, 1947).

In blood the radioactivity in the free phosphate fraction falls from 90 to 43 % between 40 min. and 2 hr. 10 min. The activity of the pineal is not changed in the corresponding fraction to any appreciable extent. The pineal is thus independent of the activity of the blood in this fraction. We have arrived at the same result by another method. A rabbit, injected with P^{32}, was killed 40 min. afterwards by compression of the thorax. Distilled water (200 ml.) was the fraction precipitable with trichloroacetic acid (phosphatides, nucleic acids) (Table 3). Two hr. after the injection this value has risen to 19 %. This confirms the findings of Fries & Chaikoff.

Obviously at least two different types of phosphate reaction take place in the brain, namely one rapid, which results in the turnover of carbohydrate esters, and one slow, which results in that of phospholipids and nucleic acids (Table 3).

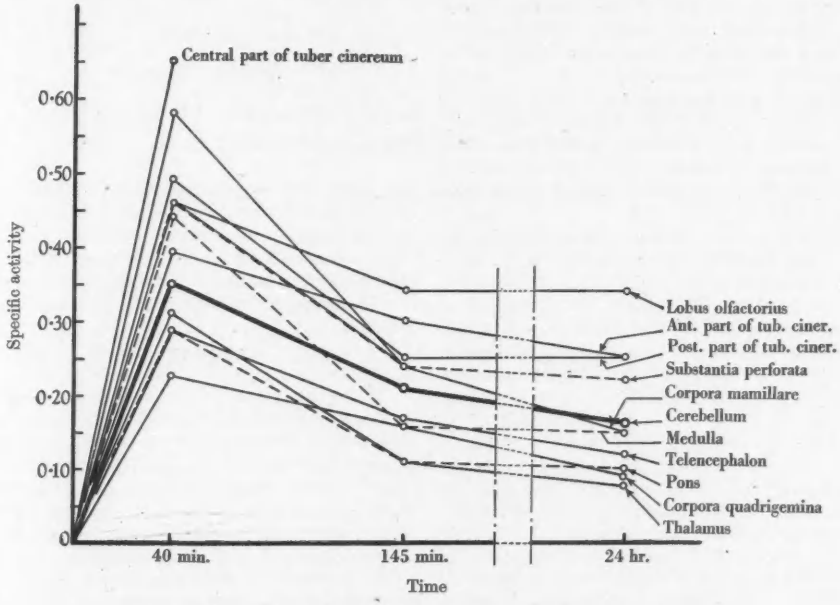

Fig. 2. Specific activity in parts of the brain with specific activity less than 1·0 at different times after the injection.

then injected slowly into both carotid arteries and was allowed to run out through the cut jugular veins. The pineal still possessed a high specific activity, even exceeding that of the control animals.

Fries & Chaikoff (1941*a*, *b*) determined the uptake of phosphate in rats of different ages with P^{32}. They divided the nervous system into four parts, namely, the forebrain, cerebellum, medulla oblongata and spinal cord. They found that the relatively highest uptake of P^{32} was that of young animals. In young animals, the radioactivity of the spinal cord exceeded that of the other parts, whereas in adults the activity of the parts of the brain predominated. They analyzed particularly the phospholipid fractions, which constituted 20–30 % of the total P^{32} in the brain after 24 hr.

Our experiments show that 3 % of the radioactivity in the pineal is recoverable after 40 min. in

In order to show that the high activity of the pineal body is not characteristic of rats only, the glands from rabbits, pigs, cats and guinea-pigs have also been studied. Table 4 shows that in all the animals investigated the activity of the pineal was at

Table 4. *Relative specific activity in the pineal body in animal species*

		Relative specific activity			
			Pituitary		
Species	No. of animals	Pineal body	Ant. lobe	Post. lobe	Blood
Rat	36	26·5	8·6	7·9	40·5
Rabbit	15	49·5	47·0	35·5	81·0
Guinea-pig	3	8·7	—	—	147·0
Pig	1	3·0	1·5	4·9	—
Cat	5	40·5	—	—	134·0

least on a level with that of the anterior and posterior lobes of the pituitary gland.

Many attempts have been made to determine the function of the pineal. Most of them yielded no valuable results and in fact disproved one another. An observation verified by several authors (MacCord & Allen, 1917; Huxley & Hogben, 1922; Addair & Chidester, 1928) is the change in colour of tadpoles fed before metamorphosis with an extract of ox-pineal. The tadpoles grew white and transparent owing to the contraction of the chromatophores. Attempts have also been made to investigate the function of the organ by extirpation, implantation and extraction experiments, and, moreover, to study possible histological changes in the pineal following the removal of other endocrine organs (cf. Bargmann 1943). Certain conditions of pubertas praecox in man have been explained as a result of a reduction or cessation in the activity of the pineal (Berblinger, 1932).

In connexion with certain surgical experiments carried out for the purpose of determining the significance of the pineal, the following observation was made which may well be quoted in this account of the introduction of P^{32} into the brain. In this experiment we studied the consequences of exoculation. It has been shown that the functional condition of the pituitary is influenced by such an operation (Bissonnette, 1937). Furthermore, Scharrer (1937) showed that the glandular cells observed by him in the diencephalon altered after the eyes had been exposed to light. A week after the removal of the eyes the experiment was performed with P^{32}. Of the different parts of the brain investigated, only the values for the medulla oblongata from the blind animals (six in number) differ from those of the normal material (48 animals) (Table 5). Statistical examination of the figures shows that there is a significant difference only between the relative specific activity of the medulla oblongata of animals with eyes removed and that of the medulla oblongata of control animals. The respective mean values are 2·48 and 1·02. The difference, 1·46, is statistically significant with $p < 0{\cdot}001$.

No accumulation of radioactive P could be observed in the pineal, the diencephalon or the pituitary. Nor did the metabolism of the corpora quadrigemina deviate from the normal value. A lower metabolism had been expected, because the anterior corpora quadrigemina contain centres which transfer impulses from the optic tract to the oculomotor nerve and thus play a part in the pupil and accommodation reflexes. These reflexes disappear after removal of the eyes, which, however, obviously entails no measurable decrease in the P^{32} content.

Table 5. *Relative specific activity in different parts of the brain of control and blind animals*

Part of brain	Control animals	Blind animals
Pineal body	26·5	18·1
Ant. lobe of pituitary	8·6	4·6
Post. lobe of pituitary	7·9	5·0
Choroid plexus	11·2	14·5
Olfactory lobe	1·30	1·55
Telencephalon	0·76	0·94
Thalamus	0·78	0·67
Ant. part of tuber cinereum	1·12	1·75
Post. part of tuber cinereum	1·20	1·17
Mammillary body	1·49	1·45
Perforated substance	1·16	2·41
Corpora quadrigemina	0·69	1·25
Cerebellum	1·0	1·00
Pons	0·72	0·57
Medulla oblongata	1·02	2·48

SUMMARY

1. The phosphate metabolism in nineteen different parts of the brain of rats has been measured with radioactive phosphorus. The pineal body has the highest specific activity. It is 3–4 times higher than that of the pituitary gland and choroid plexus. The remaining parts of the brain have a low activity. The high specific activity of the pineal body has also been found in a number of other animals.

2. All parts of the brain have a higher specific activity 40 min. after injection with radioactive P than 145 min. and 24 hr. after.

3. The high phosphate turnover in the pineal body is caused by the pineal body itself and not by blood in the organ.

4. The radioactive phosphate in the pineal changes principally in two ways: (*a*) a rapid turnover in which P^{32} enters chiefly into the carbohydrate phosphate esters; (*b*) a slow one during which P^{32} enters the phospholipids and nucleic acids.

5. After extirpation of the eyes a marked increase takes place in the phosphate turnover of the medulla oblongata.

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Antibacterial Action of Some Aromatic Amines, Amidines, Amidoximes, Guanidines and Diguanides

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The discovery of therapeutic activity in a new type of compound is a noteworthy development in chemotherapy. Such activity may be of a minor order but it can be developed by the art of the organic chemist. Discoveries of this kind often depend upon some accidental observation; it is a laborious task to search systematically, using experimental infections in animals, for specific therapeutic activity in new types of compounds. As however the vital processes of all organisms are fundamentally similar, it seems possible that by choice of a suitable organism which could be easily handled one might be able to uncover unsuspected biological activity in new types of compounds without excessive labour. For this purpose a carefully chosen group of bacterial species seems worthy of consideration.

During the war a long series of aromatic bases was prepared in connexion with investigations into the chemotherapy of virus diseases (Andrewes, King & van den Ende, 1943) and of typhus (Andrewes, King, van den Ende & Walker, 1944; Andrewes, King & Walker, 1946). As these substances were still available it was deemed worth investigating whether any possessed pronounced biological activity as revealed by bacteriostatic action on a range of bacteria *in vitro*. Such indeed proved to be the case. *p*-Methylsulphonylbenzamidine and *p*-methylsulphonylbenzylamine were found to be outstandingly active against certain obligate anaerobic bacteria. These observations were followed up and the results have been fully reported (Fuller, Tonkin & Walker, 1945; Evans, Fuller & Walker, 1944, 1945). The present communication records the results of bacteriostatic tests on a number of aromatic amines, amidines, amidoximes, guanidines and diguanides which have not hitherto been recorded.

METHODS

The methods used were similar to those previously described (Fuller, 1942). Inhibitory concentrations are recorded as mg./100 ml. (parts/100,000). The sign > means that the drug was inactive at the highest concentration tested. The tests were carried out in 200 μl. of medium contained in tubes 40 × 5 mm. (internal) which were afterwards sealed with sealing wax. For the tests with *Clostridium welchii* the broth was first boiled, reduced with 0·1% of thiolacetic acid and neutralized; 4 mm. tubes were used and these were loosely covered with glass caps and incubated in the anaerobe jar. The implant was 10 μl. of a 1:1000 dilution of an overnight culture of *Streptococcus pyogenes* or *Cl. welchii* and of a 1:10,000 dilution of the other organisms.

The effect of proteins on the activity was determined by testing the drugs against *Strep. pyogenes* in broth and in horse blood instead of in 50% serum broth as previously.

Cultures. The main tests were carried out with the following cultures: *Strep. pyogenes*, group A, type 3, 'Richards' (mouse virulent); *Staphylococcus aureus*, 'Paler'; *Cl. welchii*, 'Rosher'; *Escherichia coli*, '88'; *Proteus vulgaris*, 'Hunt'.

RESULTS

Table 1 summarizes the results obtained on a series of aromatic amidines.

It will be noticed that the aromatic amidines with few exceptions are not highly bacteriostatic. The relatively high activity of the nitro-compounds is probably due to a specific effect of the nitro-group. Of the compounds recorded in Table 1 it seems that *p*-ethoxybenzamidine (12), and *p*-sulphonamidophenylacetamidine (21), might be worth while examining on other organisms including the Protozoa. The *o*-aminophenol grouping in (18) on which Ehrlich laid considerable stress has no eutherapeutic effect in this series.

Table 1. *Inhibitory concentrations in mg./100 ml. of derivatives of benzamidine*

	In blood	In broth				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
1. Benzamidine	150	30	1000	200	750	1000
2. Benzamidine, *m*-Cl	30	50	500	8	500	500
3. Benzamidine, *p*-Cl	30	25	200	10	200	400
4. Benzamidine, *p*-Br	100	10	250	5	100	500
5. Benzamidine, *m*-NO_2	2	5	40	5	50	75
6. Benzamidine, *p*-NO_2	1	3	25	3	15	20
7. Benzamidine, *m*-NH_2	120	50	500	500	400	1000
8. Benzamidine, *p*-NH_2	100	20	150	75	200	1000
9. Benzamidine, *p*-Me	100	15	400	25	750	300
10. Benzamidine, *p*-OH	60	20	100	200	200	>1000
11. Benzamidine, *p*-OMe	50	7	150	10	250	500
12. Benzamidine, *p*-OEt	15	1	15	0·5	75	100
13. Benzamidine, 4:5-CH_2O_2	100	7	200	7	500	500
14. Benzamidine, *p*-$CONH_2$	10	5	400	100	500	>1000
15. Benzamidine, *m*-SO_2NH_2	250	50	>1000	1000	1000	>1000
16. Benzamidine, *p*-SO_2NH_2	5	1·5	500	7	1500	1200
17. Benzamidine, *p*-$CH_2SO_2NH_2$	50	25	600	500	1000	>1000
18. Benzamidine, 3-NH_2, 4-OH	50	35	300	150	500	>1000
19. Phenylacetamidine	75	100	750	150	500	1500
20. Phenylacetamidine, *p*-NO_2	30	15	75	2	20	300
21. Phenylacetamidine, *p*-SO_2NH_2	4	0·5	150	3	1000	>1000
22. α-Phenylbutyramidine	60	100	200	50	200	500

Table 2. *Inhibitory concentrations of derivatives of* p*-sulphonamidobenzamidine*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
	(*a*) Substitution in the sulphonamide group					
16. —SO_2NH_2	5	1·5	500	7	1500	1200
23. —SO_2NHMe	5	1·5	500	10	1000	>2000
24. —SO_2NHBu^{α}	50	10	300	600	200	500
25. —SO_2NMe_2	150	100	350	20	1000	>1000
26. —SO_2NEt_2	150	75	200	—	300	500
27. —$SO_2NHC(:NH)NH_2$	150	120	700	—	1000	>1000
28. —$SO_2NHCH_2C(:NH)NH_2$	35	20	500	—	1000	>1000
29. —$SO_2N(CH_2CH_2OH)_2$	200	100	>1000	>1000	1000	>1000
30. —SO_2N.CH_2.$CH_2CH_2CH_2CH_2$	100	100	200	750	500	750
31. —SO_2N.CH_2.CH_2.O.CH_2CH_2	250	150	1000	—	1000	>1000
32. —SO_2NHPh	15	5	50	20	50	200
33. —$SO_2NHC_6H_4C(:NH)NH_2$	>200	100	1000	1000	1000	>1000
34. —$SO_2NHC_6H_4.SO_2NH_2$	150	200	>1000	500	1000	1000
35. —$SO_2NHC_6H_4.SO_2NMe_2$	>50	>50	>50	>50	>50	>50
36. —SO_2NH.C.N.CH.CH.CH.CH	250	80	1000	—	1000	>1000
37. —SO_2.NHC:N.CH.CH.S	>100	60	>200	20	>200	>200
38. —SO_2NH.C:N.CMe:CH.CMe:N	>200	150	>200	>200	>200	>200
39. —SO_2NHOEt	50	50	350	15	400	1000
40. —SO_2NHOPr^{α}	150	50	120	20	250	500
41. —SO_2NHOBu^{α}	100	20	>200	15	>200	>200
	(*b*) Substitution in the amidine group					
42. —C(:NH)NHMe	200	100	>1000	500	1000	>1000
43. —C(:NMe)NHMe	200	500	>2000	1200	2000	>2000
44. —C(:NH)NH.NH_2	100	10	500	20	200	>500

The effect of substituents in the sulphonamide group and in the amidine group of *p*-sulphonamidobenzamidine, a drug which has some action on experimental typhus and a pronounced action on some bacteria is recorded in Table 2.

A survey of Table 2 shows that as a rule substitution in the sulphonamide or the amidine group reduces the bacteriostatic activity. Hydrocarbon radicals, however, increase the activity against staphylococci and the Gram-negative bacteria; e.g. the phenyl (32), diethyl (25), butyl (24), propoxy (40), and pentamethylene (30) derivatives are all more active than the parent compound. Introduction of polar groups, e.g. the conversion of a sulphonamido- (16) to a sulphaguanidino- (27) derivative, seriously lowers the activity against streptococci. In the case of the amidrazone (44), there is some enhancement of activity possibly due to a specific effect of the hydrazine grouping.

Table 3 records the results of tests on a few benzamidoximes. Comparison with the corresponding amidines in Table 1 shows that conversion to the amidoxime has an unfavourable effect, although *p*-sulphonamidobenzamidoxime is equal if not superior to *p*-sulphonamidobenzamidine in experimental typhus in mice. This superiority or equivalence is not shown to the bacteria here examined.

In Table 4 are recorded the results of the effect of substitution in the sulphonamido- or the amidoxime-group of *p*-sulphonamidobenzamidoxime.

These compounds on the whole are very weakly bacteriostatic and also sparingly soluble.

In Table 5 are given the results of tests on a series of guanidines. These compounds were specially prepared (King & Tonkin, 1946) when it was found that *p*-tolylguanidine had a marked bacteriostatic activity and also a weak true causal prophylactic activity against a sporozoite-induced infection of *Plasmodium gallinaceum* in the chick.

It will be observed that the marked anti-bacterial action of *p*-tolylguanidine is also found in *p*-ethylphenylguanidine, in *p*-anisylguanidine and in *p*-phenetylguanidine. Of these compounds *p*-anisylguanidine proved to possess the most active true causal prophylactic effect against bird malaria (King & Tonkin, 1946). There is little difference in

Table 3. *Inhibitory concentrations of derivatives of benzamidoxime*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
45. Benzamidoxime	>200	250	2000	1000	1000	1500
46. Benzamidoxime, *p*-Cl	>100	50	800	40	200	500
47. Benzamidoxime, *p*-Br	>200	20	500	20	500	500
48. Benzamidoxime, 4:5-CH_2O_2	>200	200	500	250	500	>1000
49. Benzamidoxime, *p*-SO_2NH_2	>200	50	>1000	700	1000	2000

Table 4. *Inhibitory concentrations of derivatives of* p-*sulphonamidobenzamidoxime*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
(*a*) Substitution in the sulphonamide group						
49. —SO_2NH_2 (unsubstituted)	>200	50	>1000	700	1000	2000
50. —SO_2NEt_2	>200	100	400	150	500	>500
51. —$SO_2N(CH_2.CH_2OH)_2$	>200	600	>1000	>1000	>1000	>1000
52. —SO_2N.$CH_2.CH_2.CH_2.CH_2$.CH_2	>50	100	>100	>100	>100	>100
53. —SO_2N.$CH_2.CH_2.O.CH_2$.CH_2	>200	1000	>1000	500	1000	>1000
54. —$SO_2NH.C_6H_4.SO_2NMe_2$	>100	150	>500	—	>500	>500
55. —$SO_2NH.C$:N.CH:CH.S	150	75	>500	500	>500	>500
56. —SO_2NHOH	>200	>800	>800	800	>800	>800
57. —SO_2NHOMe	300	250	250	50	200	500
58. —SO_2NHOEt	200	150	750	50	600	>1000
59. —SO_2NHOPr^{α}	>200	120	500	50	600	>1000
60. —SO_2NHOBu^{α}	150	400	250	20	>400	>400
(*b*) Substitution in the amidoxime group						
61. —C(:N.OMe).NH_2	500	250	1500	500	1000	>2000
62. —C(:N.OEt).NH_2	>200	150	700	15	>700	>700
63. —C(:NOPr$^{\alpha}$)NH_2	>200	>500	500	100	>500	>500
64. —C(:NOMe)NHOMe	>200	350	1000	800	800	>1000
65. —C(:N.OEt)NHOEt	>200	200	800	150	>800	>800

activity between the amidines of Table 1 and the guanidines of Table 5, except that the *p*-sulphonamido-derivative (85) is much less active against streptococci than is the corresponding amidine (16), and that *p*-tolylguanidine (72) is much more active than *p*-tolylamidine (9).

butyl (93) derivative when the H of the amino group is substituted.

The result of diguanide formation is shown in Table 7.

It will be noticed that the pronounced bacteriostatic effect associated with the *p*-anisyl and *p*-tolyl

Table 5. *Inhibitory concentrations of guanidines*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogens*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
66. Phenylguanidine	150	100	400	100	500	500
67. Phenylguanidine, *m*-Cl	100	20	120	100	200	200
68. Phenylguanidine, *p*-Cl	20	20	100	20	200	300
69. Phenylguanidine, *p*-Br	10	10	75	50	250	400
70. Phenylguanidine, *o*-Me	200	100	200	50	500	1000
71. Phenylguanidine, *m*-Me	20	100	150	150	200	500
72. Phenylguanidine, *p*-Me	5	0·5	10	0·5	—	300
73. Phenylguanidine, 3:6-Me_2	100	100	100	70	100	200
74. Phenylguanidine, 4:5-Me_2	50	50	50	20	50	150
75. Phenylguanidine, 4:6-Me_2	50	50	150	50	200	1000
76. Phenylguanidine, *p*-Et	1	0·5	5	0·7	70	200
77. Phenylguanidine, *p*-OH	100	30	100	50	500	2000
78. Phenylguanidine, *o*-OMe	100	200	500	500	500	—
79. Phenylguanidine, *p*-OMe	5	0·5	30	5	200	1000
80. Phenylguanidine, *p*-OEt	3	1	20	2	100	500
81. Phenylguanidine, 4:5-CH_2O_2	50	1·5	50	1·5	200	800
82. Phenylguanidine, 4:5$(OMe)_2$	20	100	200	100	500	>2000
83. Phenylguanidine, *p*-COMe	50	5	50	5	200	500
84. Phenylguanidine, *p*-SO_2OH	>250	>500	>500	>500	>500	>500
85. Phenylguanidine, *p*-SO_2NH_2	50	30	100	150	1000	>1000
86. α-Naphthylguanidine	100	20	100	50	75	150
87. β-Naphthylguanidine	60	15	50	50	50	100
88. Benzylguanidine	20	60	50	200	100	200

Table 6. *Inhibitory concentrations of derivatives (RX) of* p-*tolylguanidine* (72) *and of* p-*anisylguanidine* (79) *substituted in the guanidine group*

		In blood (mg./100 ml.)	In broth (mg./100 ml.)				
R	*X*	*Strep. pyogenes*	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
72. *p*-Tolyl	—NHC(:NH)NH_2	5	0·5	10	0·5	—	300
89. *p*-Tolyl	—N.Me.C(:NH)NH_2	100	100	500	100	100	2000
90. *p*-Tolyl	—N.Et.C(:NH)NH_2	200	50	200	150	100	800
91. *p*-Tolyl	—NH.C(:NMe)NH_2	200	150	500	50	500	1000
92. *p*-Tolyl	—NH.C(:NEt)NH_2	200	50	500	50	500	600
93. *p*-Tolyl	—NH.C(:NBu$^{\alpha}$)NH_2	100	20	100	3	100	300
94. *p*-Tolyl	—NH.C(:NH)NMe_2	500	500	1000	500	1000	>2000
95. *p*-Tolyl	—NH.C(:NH)NEt_2	300	500	2000	200	800	2000
79. *p*-Anisyl	—NH.C(:NH)NH_2	5	0·5	30	5	200	—
96. *p*-Anisyl	—NMe.C(:NH).NH_2	150	50	500	200	200	1000
97. *p*-Anisyl	—NEt.C(:NH)NH_2	100	200	200	200	100	500
98. *p*-Anisyl	—NH.C(:NMe)NH_2	150	150	300	150	700	2000
99. *p*-Anisyl	—NH.C(:NEt)NH_2	300	200	1000	100	500	1000
100. *p*-Anisyl	—NH.C(:NBu$^{\alpha}$)NH_2	100	50	200	10	200	300
101. *p*-Anisyl	—NH.C(:NH)$NHPr^{\beta}$	—	50	600	100	500	1000
102. *p*-Anisyl	—NH.C(:NH)NEt_2	200	800	2000	200	1000	>2000
103. *p*-Anisyl	—NH.C(:NMe)NHMe	500	500	2000	500	1500	>2000
104. *p*-Anisyl	—NMe.C(:NMe)NHMe	2000	500	1000	500	1000	1000

Table 6 shows that substitution in the guanidine group both in the case of *p*-tolyl- and *p*-anisylguanidine leads to diminished bacteriostatic activity. There is, however, an increase in activity, especially against *Cl. welchii*, from the methyl (91) to the and homologous groups observed in the guanidines is lost here and that in general the diguanides are not markedly bacteriostatic.

When the N^1 nitrogen atom of the diguanides is alkylated (Table 8), there is no pronounced change

Table 7. *Inhibitory concentrations of diguanides*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
105. Phenyldiguanide	30	5	400	100	1000	>1000
106. Phenyldiguanide, *m*-Cl	100	20	60	50	100	200
107. Phenyldiguanide, *p*-Cl	70	50	50	30	100	100
108. Phenyldiguanide, *p*-Br	30	20	30	30	75	50
109. Phenyldiguanide, *o*-Me	200	200	500	100	500	1000
110. Phenyldiguanide, *m*-Me	200	100	100	70	200	500
111. Phenyldiguanide, *p*-Me	100	200	100	70	200	500
112. Phenyldiguanide, 3:6-Me_2	200	100	100	70	200	500
113. Phenyldiguanide, 4:5-Me_2	100	50	50	8	100	200
114. Phenyldiguanide, 4:6-Me_2	200	100	100	100	200	500
115. Phenyldiguanide, *p*-Et	100	50	30	4	50	120
116. Phenyldiguanide, *p*-OH	100	100	200	300	500	1500
117. Phenyldiguanide, *o*-OMe	200	500	500	200	500	2000
118. Phenyldiguanide, *p*-OMe	100	100	200	100	500	1500
119. Phenyldiguanide, *p*-OEt	100	10	50	100	200	500
120. Phenyldiguanide, 4:5-CH_2O_2	100	75	120	70	400	1000
121. Phenyldiguanide, *p*-COMe	200	100	200	100	200	700
122. α-Naphthyldiguanide	50	10	75	50	30	120
123. β-Naphthyldiguanide	50	10	50	20	50	100
124. Benzyl diguanide	50	70	50	100	100	500

Table 8. *Inhibitory concentrations of various substituted diguanides*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
125. N^1-Methyltolyldiguanide	80	20	50	150	250	1500
126. N^1-Ethyltolyldiguanide	100	40	50	250	100	500
127. N^1-Methyl *p*-methoxyphenyldiguanide	200	30	200	1000	500	2000
128. N^1-Ethyl *p*-ethoxyphenyldiguanide	100	120	50	500	150	800
129. *p*-Chlorophenyl-N^5-methyl-N^5-*iso*propyldiguanide	15	5	20	10	25	70

Table 9. *Inhibitory concentrations of aniline and derivatives*

	In blood (mg./100 ml.)	In broth (mg./100 ml.)				
	Strep. pyogenes	*Strep. pyogenes*	*Staph. aureus*	*Cl. welchii*	*Esch. coli*	*Proteus vulgaris*
130. Aniline	>200	150	500	200	600	600
131. Aniline, *m*-Cl	200	75	200	10	200	150
132. Aniline, *p*-Cl	—	100	200	10	200	150
133. Aniline, *p*-Br	>100	50	100	50	100	75
134. Aniline, *p*-NO_2	75	7	100	100	100	100
135. Aniline, *m*-NH_2	100	35	200	20	>1000	1000
136. Aniline, *p*-NH_2	50	1·5	25	20	100	20
137. Aniline, *p*-NHCOMe	>200	100	>1000	—	>1000	>1000
138. Aniline, *p*-Me	>200	1·5	200	200	400	500
139. Aniline, *p*-Et	200	30	300	500	200	400
140. Aniline, *p*-OH	>50	1·5	5	200	20	5
141. Aniline, *o*-OMe	500	100	200	100	500	500
142. Aniline, *p*-OMe	200	20	100	200	100	1000
143. Aniline, *p*-OEt	200	3	200	500	300	500
144. Aniline, *p*-SO_2NH_2	4	—	10*	—	4*	7*
145. Aniline, *p*-COOH	1000	1000	1000	1500	750	1500
146. Benzylamine	20	100	600	—	200	700

* In synthetic medium.

in bacteriostatic properties but if the terminal N^5 nitrogen atom is alkylated as in *p*-chlorophenyl-N^5-methyl-N^5-*iso*propyldiguanide (M4430) (for which I am indebted to Imperial Chemical Industries (Pharm.), Ltd.) there is a distinct enhancement of bacteriostatic properties. It is significant that this substance is closely related to paludrine and like paludrine has pronounced antimalarial properties (Curd & Rose, 1946).

Finally, in Table 9, are summarized the effects of substitution in the nucleus of a series of anilines. As might be anticipated, *p*-phenylenediamine and *p*-aminophenol, substances readily oxidized to imines, are markedly active but *m*-phenylenediamine is not. *p*-Aminobenzoic acid is relatively harmless whilst sulphanilamide is uniformly of marked activity.

Most of the substances were also tested against *Pseudomonas aeruginosa* which was inhibited on an average by about three-quarters of the concentration necessary for *Proteus vulgaris*. A few were tested against *Eberthella typhosa* which was inhibited at approximately the concentration required for *Esch. coli* and against *Shigella paradysenteriae* Flexner W which needed approximately three-quarters of that value.

DISCUSSION

Most of the compounds are moderately active against streptococci, less so against *Cl. welchii*, and weakly active against staphylococci and the Gram-negative bacteria. This is the common pattern of activity for simple bases. *p*-Sulphonamidobenzamidine (16) is very similar in activity to the German drug 'Marfanil' (*p*-sulphonamidobenzylamine), and like it has a strong basic group separated from the benzene ring by a carbon atom. The insertion of a second carbon atom to give *p*-sulphonamidophenylacetamidine (21) results in even greater potency. The corresponding compound in the Marfanil series (*β*-*p*-sulphonamidophenylethylamine) was shown by Lawrence (1945) to be inactive, and Fuller, Tonkin & Walker (1945) found that *β*-*p*-methylsulphonylphenylethylamine was very much weaker than *p*-methylsulphonylbenzylamine. The activity of the amidines persists through a greater change of constitution than does that of the amines.

In general, substitution, particularly by polar groups, lowers activity, which means that there are few outstanding drugs in the present series. However, alkyl and phenyl substitution in *p*-sulphonamidobenzamidine and -amidoxime raises the activity against *Esch. coli* and *Proteus vulgaris* (24, 26, 32, 50); and *p*-methyl, -ethyl or -ethoxyl cause a general increase in the activity in the guanidine and aniline series (12, 72, 76, 80, 138, 143). *Para* substitution, particularly of a sulphonamide or a carbamide group often raises the activity against streptococci specifically (14, 16, 21, 49, 144), while *meta* substitution lowers it (2, 7, 15).

Although the first substituent may lower the activity, higher homologues may be more active than the first member. For example, there is always an increase in activity from *p*-OH to *p*-OC_2H_5 (10, 11, 12; 77, 79, 80; 116, 118, 119). Similarly, as the series of O-ethers of *p*-sulphonamidobenzamidoxime is ascended (56–60), the activity against *Cl. welchii* increases. Changes in constitution affect the activity against streptococci and *Cl. welchii* more than that against other bacteria (e.g. 16, 17). The staphylococci are very similar to the Gram-negative bacteria in their response to changes in constitution.

The activity of most of the drugs is decreased in blood. In general those groups which lead to an increase of activity also show a more serious interference by blood.

SUMMARY

1. The bacteriostatic activities of a number of derivatives of benzamidine, phenylguanidine, phenyldiguanide and benzamidoxime are recorded.

2. Substitution in these compounds generally leads to lowering of activity, but a methyl, ethyl, methoxyl, ethoxyl or sulphonamide group in the *para* position greatly increases the potency against streptococci.

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The Hyaluronidase Content of Semen

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The occurrence in mammalian testes and aqueous extracts of spermatozoa of a substance causing increased spread of fluids injected intradermally was described by Hoffman & Duran-Reynals (1931) and McClean (1930, 1931). These observations have since been confirmed by numerous workers (Chain & Duthie, 1940; McClean, 1943; Humphrey, 1943) and the 'spreading factor' has been shown to be identical with the enzyme hyaluronidase first described by Meyer, Dubos & Smythe (1937). The work of Long (1912), Yamane (1930), and Pincus & Enzmann (1935) had demonstrated that a substance present in semen had the property of dissolving the viscous gel cementing the cumulus cells around freshly ovulated rat ova, and the implication of testicular hyaluronidase in the fertilization process was suggested by McClean & Rowlands (1942) who demonstrated its ability to perform the same function, a finding subsequently confirmed by Fekete & Duran-Reynals (1943) for the mouse and by Leonard & Kurzrok (1945) for the rat. Rowlands (1944) observed an increase in the fertilizing capacity of dilute rabbit sperm suspensions on the addition of an hyaluronidase-containing medium. The existing evidence, therefore, suggests that the role of seminal hyaluronidase in the fertilization process consists in denuding the egg of its surrounding follicle cells so as to enable penetration and syngamy to be effected by a spermatozoon, and that the ineffectiveness of oligospermic semen may be accounted for, at least in part, by its inability to establish, in the vicinity of the egg, a sufficient concentration of the enzyme to dissolve off the cumulus.

In order to investigate this hypothesis further, and also to provide data which might serve as a basis for the possible therapeutic use of hyaluronidase in the treatment of infertility due to oligospermia, it was thought useful to make some quantitative investigations of the hyaluronidase content of the semen from different species and individuals. This paper sets forth some of the results which have been obtained.

MATERIAL AND METHODS

(1) *Human semen.* Fifty-eight human semen samples have been assayed. They were obtained either by coitus interruptus followed by ejaculation into a glass jar or by masturbation; no condom specimens have been used. In order to prevent loss of potency of the contained enzyme they were stored, as soon as possible after being produced, in a refrigerator, and after sperm counts had been made, in the frozen state. For assay, they were thawed, diluted with 0·5% gum arabic in the proportion of 1 ml. semen to 3 ml. gum arabic (or higher dilutions in the case of very dense specimens) and filtered through cotton wool to remove particles which might have blocked the viscosimeter tubes.

(2) *Rabbit semen.* Eleven rabbit semen samples have been assayed. They were obtained by the artificial vagina technique. For assay, 0·1 ml. was diluted with 9·9 ml. water and allowed to stand at 0° for at least 24 hr. in order to allow the release of all the hyaluronidase from the spermatozoa (Swyer, 1947). If some time was to elapse before viscosimetric assay, the suspensions were stored frozen at −10°. Further dilutions were made to the required degree with 0·5% gum arabic.

(3) *Boar semen.* Six boar semen samples, which had been collected by the artificial vagina technique and freeze-dried in bulk at the end of 1944 and beginning of 1945, have been assayed. They were reconstituted to the original volume by addition of the appropriate quantities of water, sperm counts being made on the reconstituted semen. For assay, they were diluted, if necessary, with 0·5% gum arabic.

(4) *Bull semen.* Five samples of bull semen, obtained by the artificial vagina technique, have been assayed. The first two were pooled samples of several ejaculates. They were stored frozen until required when they were thawed and diluted with suitable amounts of 0·5% gum arabic for assay.

(5) *Dog semen.* Seven samples of dog semen, collected by the artificial vagina technique, have been tested, some of them after storage in the frozen state and others within a few hours of ejaculation.

(6) *Fowl semen.* Ten samples of fowl semen, obtained by manual stimulation have been tested. Sperm counts and viscosimetric testing were done on the first two samples while fresh. The remainder were stored frozen, after which treatment it was, unfortunately, found impossible to do sperm counts with any accuracy because of agglutination of many of the spermatozoa; there is, however, no reason to believe that tests for hyaluronidase were vitiated by this procedure.

Sperm counts were made in a Spencer Bright Line haemocytometer, the dilutions, made with haemocytometer pipettes, being adjusted so that in general 200–400 sperms were counted for each sample. Hyaluronidase assays were made by the viscosimetric method described by Swyer & Emmens (1947), in which the unit of hyaluronidase activity is approximately equal to 53·5 viscosity reducing units of McClean & Hale (1941). The standard error of this method is less than 10%.

RESULTS

(1) *Human semen.* The hyaluronidase contents, expressed in units/ml. (Swyer & Emmens, 1947) and in units/total ejaculate, of fifty-eight samples of human semen are shown in Table 1. It can be seen that the four azoöspermic specimens were totally devoid of hyaluronidase, and that with increasing sperm density there is an increasing hyaluronidase concentration. Analysis of these figures for the linear regression of enzyme content on sperm density yields a correlation coefficient of 0·942, and a regression coefficient of 0·003369, indicating that 88·7 % of the hyaluronidase content can be accounted for by the sperm density. The equation for the regression line is $y = 0{\cdot}003369x + 0{\cdot}024545$, where y = hyaluronidase content in units/ml. and x = sperm count in millions/ml. (see Fig. 1).

Table 1. *Sperm count and hyaluronidase content of human semen*

Sperm count (millions/ml.)	Volume of ejaculate (ml.)	Hyaluronidase (units/ml.)	Hyaluronidase units in total ejaculate
0	1·4	0	0
0	3	0	0
0	3·2	0	0
0	1·3	0	0
0·08	2·5	Very faint trace	—
c. 0·5	2·2	Trace	—
1·5	2	0·017	0·025
2·3	5	0	0
2·5	2·5	0·05	0·125
2·7	7	0·038	0·266
3	3·5	0·027	0·095
6·2	0·8	Trace	—
7	3·6	0·093	0·335
9·5	5	0·05	0·25
12	3	0·08	0·24
12	2	0·108	0·22
14	6	0·051	0·306
14	5·5	0·042	0·23
15	2·7	Trace	—
17	6·2	0·14	0·87
23	2·7	0·08	0·27
28	2·7	0·15	0·4
28	2·5	0·116	0·29
33	6·6	0·077	0·51
40	3·5	0·058	0·202
40	2·5	0·32	0·8
45	2	0·28	0·56
48	3·2	0·1	0·32
52	2·4	0·19	0·45
54	1·6	0·36	0·575
61	5·8	0·066	0·38
65	3·5	0·46	1·62
67	1·7	0·365	0·62
80	1·7	0·16	0·27
80	5·6	0·395	2·2
92	1·8	0·425	0·765
97	1·8	0·313	0·536
100	6·0	0·41	2·46
115	3·8	0·364	1·385
120	1·8	0·41	0·74
125	1·2	0·68	0·815
140	5	0·33	1·65
140	2·6	0·534	1·39
145	1·5	0·23	0·34
145	2·5	0·55	1·375
160	2·5	0·29	0·72
160	1·5	0·23	0·32
160	6·3	0·415	2·62
165	4	1·0	4·0
180	6·3	0·99	6·25
185	4	0·49	1·96
200	3·1	0·86	2·67
235	2·5	0·735	1·84
275	2·8	1·015	2·84
280	2·3	1·06	2·44
320	3·8	1·15	4·37
460	2·4	1·62	3·9
490	1·3	1·57	2·04

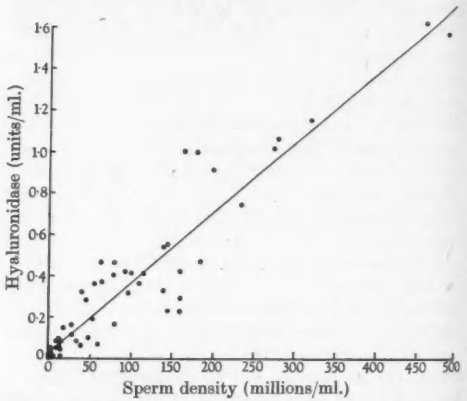

Fig. 1. Relationship between sperm density and hyaluronidase content in human semen.

It may therefore be concluded that the hyaluronidase content of human semen is almost entirely dependent on the sperm count, since the combined errors of assaying and counting would easily account for the remaining 11·3 % of variation. A further corollary is that the output of hyaluronidase/sperm in different individuals must be remarkably constant. It may be pointed out that in this series, which is not a random sample from a normally distributed population, the correlation coefficient is scarcely permissible; the use of the regression function, on the other hand, is not affected by the nature of the series and so is preferable.

From Fig. 1 it can be seen that the expected hyaluronidase content of a human semen sample containing 100 million sperms/ml. is about 0·38 unit. With an average volume of 3 ml. this would give 1·08 units in the total ejaculate.

(2) *Rabbit semen.* The hyaluronidase contents of eleven samples, the densities of which ranged from 120 to 1150 million sperms/ml. are shown in Fig. 2 and range from 39 to 254 units/ml. The close correlation between sperm count and enzyme content is evident.

The hyaluronidase contents of a few rabbit testes were also estimated by grinding with sand, extracting with water in the cold for 24 hr., filtering and assaying the filtrate. One buck, whose ejaculates obtained weekly for 15 weeks were found to contain from 115 to 820 million sperms/ml., had a unilateral congenitally undescended testicle. The normal testis had a moist weight of 1·94 g. and contained 62·5 units of hyaluronidase (31·7 units/g. moist tissue). The undescended testis weighed 0·23 g. and contained no demonstrable hyaluronidase. The two testes of a 27-day-old male (they were as yet undescended) weighed 45 mg. each and also contained no demonstrable hyaluronidase.

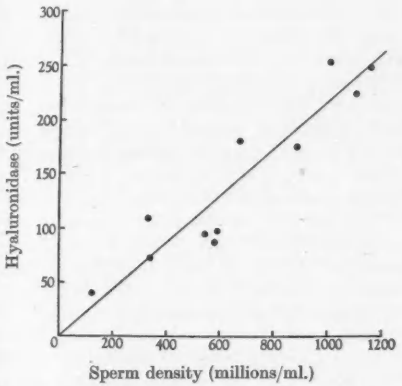

Fig. 2. Relationship between sperm density and hyaluronidase content in rabbit semen. (In this and subsequent figures, the straight line drawn through the origin is not a fitted regression line.)

(3) *Boar semen.* The hyaluronidase contents of six samples, the densities of which ranged from 120 to 390 million sperms/ml., are shown in Fig. 3, and are seen to range from 0·24 to 0·77 unit/ml. With the exception of one specimen, a very close correlation between sperm count and hyaluronidase content can be seen.

(4) *Bull semen.* The hyaluronidase contents of five samples, the densities of which ranged from 300 to 1320 million sperms/ml., are shown in Fig. 4, and range from 22 to 94 units/ml. Again, a close correlation between sperm count and hyaluronidase content is evident.

(5) *Dog semen.* Of the seven samples of dog semen tested for hyaluronidase activity, sperm counts were made on only four. In three of these, the sperm counts were 4, 155 and 300 millions/ml. and no hyaluronidase activity could be detected. In the fourth, there were 130 million sperms/ml. and 0·28 unit of hyaluronidase/ml. Of the remaining three samples whose densities were not estimated, one contained a trace of hyaluronidase and the others 0·015 and 0·025 unit respectively. (Both the latter specimens were able to effect the disintegration of the cumulus surrounding freshly ovulated rabbit ova in the course of about 1 hr. at room temperature.) The variability in results cannot be ascribed to the treatment which the semen samples had received

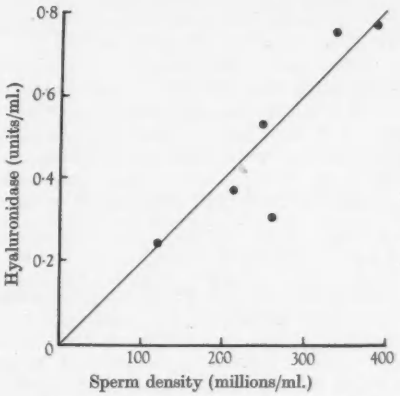

Fig. 3. Relationship between sperm density and hyaluronidase content in boar semen.

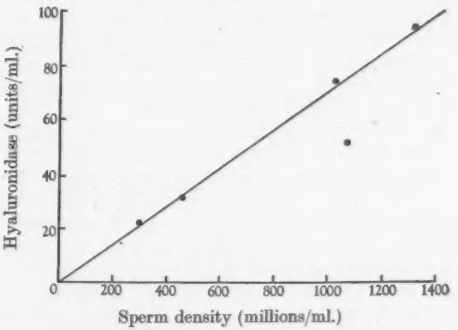

Fig. 4. Relationship between sperm density and hyaluronidase content in bull semen.

since hyaluronidase activity and inactivity were found among the fresh as well as the frozen specimens. It is evident, therefore, that the hyaluronidase content of dog semen is variable independently of the sperm density, is of a low order, and in some cases at least, is too small to be detected by the method of assay used.

(6) *Fowl semen.* Of the ten samples tested for hyaluronidase activity, six were found to show none at all. In the remaining four it was impossible to make any attempt at accurate assay since the degree

of activity and the quantity of semen available in each case was insufficient to perform the necessary procedures. A very rough estimate of activity would assign to the four samples hyaluronidase contents of 1, 0·5, 0·3 and 0·05 unit/ml. respectively. The only two samples on which sperm counts were made contained 4600 and 2500 million sperms/ml., and both showed no hyaluronidase activity (they were not frozen before testing, but since four of the frozen samples also showed no hyaluronidase activity, it is not likely that freezing would have led to a different result). The densities of those specimens possessing viscosity-reducing activity were in no case greater and in some far less (probably as low as 10 million sperms/ml. in one) than these. It appears, therefore, that fowl semen contains little or no hyaluronidase and that no correlation between hyaluronidase activity and sperm density exists in this species.

DISCUSSION

The number of sperms necessary to fertilize a mammalian ovum is apparently very great, and is considerably larger than would appear to be sufficient merely to ensure the chance contact of at least one sperm with the egg. The suggestion of McClean & Rowlands (1942) that the presence of a large number of sperms in the vicinity of the egg may be necessary to ensure the production of concentration of hyaluronidase sufficient to liquefy the hyaluronic acid gel cementing the follicle cells around the egg, assumes that the hyaluronidase content of semen is related to its sperm density. The evidence presented in this paper justifies this assumption and thereby lends considerable support to the above-mentioned theory. Pincus (1930) and Pincus & Enzmann (1932) have shown that while spontaneous dissolution of the cumulus takes place as the unfertilized ovum descends the uterine tube, this process requires some 6–8 hr. to reach completion in the rabbit, by which time the egg has acquired a layer of protein sufficiently thick to prevent penetration by spermatozoa. The hyaluronidase mechanism, therefore, enables fertilization to occur within 2 or 3 hr. of ovulation, while the deposition of protein around the unfertilized ovum prevents fertilization of ageing eggs.

With further regard to the direct proportionality between sperm density and hyaluronidase content in mammalian semen, some evidence to this effect, in the case of man, had already been provided by Joël & Eichenberger (1945) and by Werthessen, Berman, Greenberg & Gargill (1945), though at the time the work reported here was done these papers were unknown to the author. Since azoöspermic human semen is devoid of hyaluronidase, while the regression line of enzyme content on sperm density passes through zero, it follows that no hyaluronidase is supplied by the accessory gland secretions. The intimate association of the enzyme with the spermatozoa, which is further borne out by the results reported in an accompanying paper (Swyer, 1947), and the evidence relating to the hyaluronidase contents of active and inactive rabbit testes, may therefore be regarded as establishing beyond reasonable doubt the origin of seminal hyaluronidase.

No explanation can at present be advanced to account for the anomalous results obtained in the case of dog semen.

Of considerable interest is the finding that hyaluronidase was absent from six out of ten samples of fowl semen and present in the remaining samples in amounts which, considering their sperm densities, must be regarded as trivial. In view of the fact that the ova of birds are not surrounded by cumuli (see, for example, Brambell, 1925), this observation accords with the hypothesis concerning the presence and function of seminal hyaluronidase. The observation reported by Hamilton & Laing (1946), that cumulus cells persist around the unfertilized ovum of the cow for 9–14 hr. after ovulation clears up the discrepancy which had appeared to exist in this species by reason of the belief that the ovum of the cow was devoid of a cumulus at the time of ovulation.

Attention may be drawn to the striking differences in the absolute values for the hyaluronidase contents of semen in different species, when expressed in terms of standard sperm densities, in contrast to the uniformity among individuals of the same species. Thus, the hyaluronidase contents/100 million spermatozoa/ml., of the semen of rabbit, bull, man and boar are approximately 20, 7, 0·4 and 0·2 units, respectively. It seems probable that a fairly close relationship exists between the numbers of spermatozoa necessary for fertility in different species and the hyaluronidase contents of the spermatozoa.

From the standpoint of the therapeutic aspects of the problem of infertility, the function of hyaluronidase in the fertilization process suggests the possibility of treating infertility in humans due to oligospermia by supplementing the deficiency in enzyme of the semen with added hyaluronidase. On the other hand, it may be pointed out that the existence of a form of sterility due to quantitative deficiency of hyaluronidase in semen which is otherwise normal in all respects (sperm density, morphology, motility and viability) is rendered very unlikely by the close correlation which has been shown to exist in human semen between hyaluronidase content and sperm density.

SUMMARY

1. Assays of the hyaluronidase content of the semen of men, rabbits, bulls, boars, dogs and fowls have been made by the method described by Swyer & Emmens (1947). These have shown that in the

first four species there is a close correlation between hyaluronidase content and sperm density. In the case of dogs and fowls no such correlation could be found.

2. The absence of hyaluronidase in a cryptorchid and in prepubertal rabbit testes is demonstrated. The conclusion is reached that seminal hyaluronidase originates in active seminiferous epithelium and is not secreted by the accessory glands.

3. The bearing of these results on the role of hyaluronidase in fertilization and its implication in the possibility of treatment of infertility due to oligospermia is discussed.

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The Release of Hyaluronidase from Spermatozoa

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The belief that seminal hyaluronidase is associated with spermatozoa rather than with the seminal plasma is suggested by the close correlation between hyaluronidase content and sperm density in several species of mammals; by the absence of hyaluronidase from azoöspermic semen; and by its presence in testes in which active spermatogenesis is taking place and its absence in inactive testes (prepubertal and cryptorchid), as shown in an accompanying paper (Swyer, 1947). The present report is concerned with some of the factors which appear to be involved in the liberation of the hyaluronidase contained in spermatozoa.

EXPERIMENTAL

Material and methods. All the experiments to be described were carried out with rabbit semen obtained by the artificial vagina technique. Hyaluronidase assays were made by the method of Swyer & Emmens (1947), the unit of hyaluronidase activity being that described by these authors. The standard error of this method is less than 10%.

Extraction of hyaluronidase with water

McClean (1931) showed that extracts of spermatozoa made with distilled water contain large amounts of the 'spreading factor' now known to be hyaluronidase. It was observed, however, that if an aqueous suspension of spermatozoa were assayed within a short time of being made up, the hyaluronidase value obtained was less than if the suspension had been allowed to stand for some hours before assaying.

The following experiment, therefore, was performed to investigate this point. Three pooled ejaculates were used; four suspensions were made by adding 0·2 ml. semen to 9·8 ml. distilled water. A fifth portion of 0·2 ml. semen was freeze-dried in an ampoule. The five samples of semen were then subjected to the treatment shown in Table 1 and assayed for hyaluronidase. The values, expressed in units/ml. of semen show that a short period of freezing at $-10°$, or a considerable period of standing at 0° enabled an apparently maximal amount of

Table 1. *Effect of various treatments on extraction of hyaluronidase from aqueous suspensions of spermatozoa*

Suspension	1	2	3	4	5
Treatment	Assayed within 40 min.	Frozen for 3 hr. at −10° before assay	24 hr. at 0° before assay	Frozen for 6 days before assay	Freeze-dried: assayed 6 days later
Hyaluronidase (units/ml.)	41·5	89·5	84·5	83·5	82

hyaluronidase to pass into solution. It can also be seen that prolonged freezing resulted neither in increased extraction nor in significant loss of potency of the extract, while freeze-drying destroyed only a small fraction of the potency. Freeze-dried semen preparations, it may be mentioned, have been found to suffer no change of potency over periods exceeding 6 months.

In order to test whether freezing the semen suspension is sufficient to extract all the available hyaluronidase, a further suspension, made up as above, was frozen for 24 hr., thawed and centrifuged. The supernatant was removed and the deposit of sperms resuspended in 5 ml. water and immediately centrifuged for 10 min. The supernatant was discarded and the sperms ground up with sand, shaken with 5 ml. water and filtered. The filtrate was then tested for hyaluronidase activity, but none was found, suggesting that extraction by freezing was complete.

Liberation of hyaluronidase in Baker's solution

The foregoing experiments showed that hyaluronidase was liberated from dead sperms in aqueous suspension. The question then arose whether living sperms could liberate hyaluronidase, and for this purpose, sperm suspensions in Baker's (1931) solution were studied. In the first place, it was found that when samples (0·2 ml.) of semen were suspended in equal quantities (9·8 ml.) of water or Baker's solution, frozen, thawed and assayed, no significant difference in the hyaluronidase values was obtained (87·5 and 86 units/ml. semen respectively). Baker's solution, therefore, does not appear to interfere with hyaluronidase assay.

In order to compare the liberation of hyaluronidase in a water suspension with that in Baker's solution suspension in which the sperms remain alive, the experiment recorded in Table 2 was performed. From four pooled ejaculates, two suspensions containing 0·2 ml. semen in 4·8 ml. water, and a third containing 0·2 ml. semen in 4·8 ml. Baker's solution were made. One of the aqueous suspensions was left at 0° for 24 hr. and on assay yielded 87·5 units/ml. semen. The other two suspensions were treated as shown in Table 2, the spermatozoa in each instance being separated by centrifuging for 10 min. (the times indicated in the table include this period of centrifuging).

It will be seen from Table 2 that there was no significant difference between supernatants 1*a* and 1*b*, from which it was thought that the value obtained represented, for the most part at any rate, that of the hyaluronidase actually in solution in the plasma. (A subsequent experiment, described below, would suggest that these values also include some hyaluronidase liberated from the spermatozoa.) The hyaluronidase contents of supernatants 2*a*, 2*b*, 3*a* and 3*b* show that, under similar circumstances, the enzyme is liberated in significantly larger amounts in aqueous than in Baker's solution suspensions. The results of the whole experiment also confirm the belief that the major part (at least $\frac{3}{4}$) of the hyaluronidase of semen as ejaculated is associated with the sperm fraction, while the remainder must be regarded as having passed from the spermatozoa into solution in the plasma.

Table 2. *Hyaluronidase contents of supernatants from suspensions of spermatozoa (a) in water and (b) in Baker's solution. Supernatants removed at times shown, the sperm deposits in each case being resuspended in fresh water or Baker's solution as before, reconstituting the original volumes of suspensions*

Supernatant	Time of removal from suspension (min.)	Hyaluronidase (units/ml. semen) Aqueous (*a*)	Hyaluronidase (units/ml. semen) Baker's solution (*b*)
1	10	21	21·5
2	160	18·5	9·25
3	120	11	6
Total	290	50·5	36·75

To see whether the results obtained in the above experiment gave an indication of the rate of release of hyaluronidase, a suspension of spermatozoa was made by adding 0·2 ml. semen (not the same as that used in the previous experiment) to 19·8 ml. Baker's solution from which successive portions were removed at intervals and centrifuged for 10 min. The supernatants were separated and assayed. The supernatant of the first portion, removed after 5 min., yielded 18·5 units of hyaluronidase/ml. semen; that of the second, removed after 25 min., 17·5 units; that of the third, removed after 50 min., 18 units; and that of the fourth, removed after 80 min., 19 units. There is no significant difference in these values, whence it may be concluded that the amount of hyaluronidase which passes into solution in a suspension of live spermatozoa in Baker's solution is the same whether the sper-

matozoa remain in contact with the suspending medium for 15 min. or for 90 min. The results of the previous experiment, therefore, do not indicate the rate of liberation of hyaluronidase.

Absence of hyaluronidase production by spermatozoa

To investigate whether live spermatozoa produce hyaluronidase, or whether they merely liberate the preformed enzyme, three pooled ejaculates were used. Successive 0·2 ml. portions were suspended in 4·8 ml. water and in two similar volumes of Baker's solution. The aqueous suspension was immediately frozen. One of the Baker's solution suspensions was then heated to 56° until all the sperms were killed (30 min.) after which it and the other Baker's solution suspension were left for 24 hr. at room temperature, by which time the sperms in the unheated suspension were sluggishly motile. Both suspensions were then frozen for 24 hr. Hyaluronidase assays gave values of 66, 60 and 56 units/ml. semen for the aqueous, unheated and heated Baker's solution suspensions respectively. Over the period of 24 hr., therefore, no detectable production of hyaluronidase occurred. The lower value of the heated suspension may be ascribed to some destruction of enzyme by the heating.

Effect of concentration of spermatozoa on liberation of hyaluronidase from spermatozoa suspended in Baker's solution

Portions (0·1 ml.) of three pooled ejaculates were diluted with Baker's solution to make suspensions of 4, 8 and 16 ml. respectively. After 1 hr. the suspensions were centrifuged, and the supernatants removed and assayed. Two other similar experiments were performed, the volumes of suspension in these being 3, 6, 12 and 24 ml. respectively. The results are shown in Fig. 1. When the quantities of hyaluronidase liberated are plotted against the logarithm of the volume of suspension, it can be seen that over the range of volumes used there is a close approximation to a straight line relationship. This is an indication that the liberation of hyaluronidase from live spermatozoa may follow fairly simple physico-chemical laws.

It now became of interest to see whether the presence of hyaluronidase in the suspending medium influenced the extent to which the enzyme was liberated from spermatozoa placed in such a medium. Accordingly, a solution of hyaluronidase in Baker's fluid was prepared by adding 1 ml. semen to 24 ml. Baker's solution, freezing the suspension to liberate the enzyme followed by thawing and centrifuging. To 19·8 ml. of the supernatant 0·2 ml. of semen was added, while an equal quantity of semen was suspended in a further 19·8 ml. of Baker's solution. After 50 min. both suspensions were centrifuged for 10 min., the supernatants were discarded and to each deposit 10 ml. of water were added, the sperms being stirred up and the suspension then frozen to liberate the remaining hyaluronidase. The suspensions were then assayed the results being 30·5 and 23 units/ml. semen respectively, thus indicating that 7·5 units/ml. of hyaluronidase were retained by the spermatozoa which had been suspended in the hyaluronidase-containing Baker's solution.

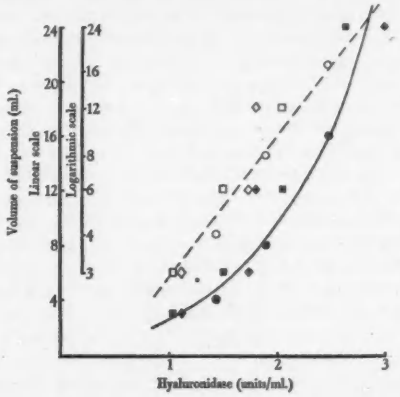

Fig. 1. Relationship between quantity of hyaluronidase liberated from 0·2 ml. semen suspended in Baker's solution, the volume of suspension (•—•), and the logarithm of the volume of suspension (○ - - - ○). The different symbols refer to the three experiments. (The straight line is not a fitted regression line.)

Effect of hyaluronate and heparin on liberation of hyaluronidase from spermatozoa

On the assumption that the cement substance of the cumulus surrounding freshly ovulated mammalian eggs is composed of hyaluronic acid (McClean & Rowlands, 1942) and that a loose combination between enzyme and substrate might lower the effective concentration of the latter in the vicinity of the egg, it seemed possible that the presence of hyaluronate might accelerate the liberation of hyaluronidase from spermatozoa. To investigate this point, and at the same time to investigate what effect heparin, a hyaluronidase inhibitor (McClean, 1942; Rogers, 1946), might have on hyaluronidase liberation, the following experiment was carried out.

Two pooled ejaculates were used and three suspensions were made by adding successive portions of 0·3 ml. semen to 9·7 ml. amounts of Baker's fluid. One of the suspensions was made to contain 10 mg. heparin (1000 Toronto units) and another 0·1 g. potassium hyaluronate. The activity of the sperms was found to be equal in all three suspensions at the start of the experiment. The suspensions were then

placed in a 37° incubator for 1½ hr., at the end of which time sperm activity was still approximately equal, although there was a good deal of head agglutination in the suspension containing hyaluronate. The suspensions were centrifuged for 10 min., when the deposit in the plain Baker's solution was resuspended in its own supernatant, while the other two supernatants were discarded, physiological saline being added to the original level, and the deposits stirred up. The suspensions were frozen to liberate the hyaluronidase remaining in the sperms. Assay of the plain Baker's solution suspension yielded 43·5 units of hyaluronidase/ml. semen; of the heparin-containing suspension 29·5 units/ml.; of the hyaluronate-containing suspension, 33 units/ml. These latter two values are not significantly different, and indicate that between one-quarter and one-third of the total hyaluronidase has passed into solution. Since this is about the expected amount which would have entered solution in a plain Baker's solution suspension it must be concluded that the presence neither of hyaluronate nor of heparin has any appreciable effect on the liberation of hyaluronidase from spermatozoa.

DISCUSSION

The experiments described above indicate the conditions under which apparently complete extraction of seminal hyaluronidase can be achieved—namely, freezing for a short period or suspension in water for some 24 hr. at 0°. They demonstrate, moreover, the possibility of extracting hyaluronidase from washed spermatozoa and, by showing that at least three-quarters of the total hyaluronidase content of semen as ejaculated is associated with the spermatozoa, confirm the supposition that hyaluronidase production is a function of the seminiferous epithelium and not of the accessory glands. From the point of view of the function of seminal hyaluronidase, if this is conveyed by the spermatozoa to the vicinity of the freshly ovulated egg, there to effect disintegration of the cumulus (McClean & Rowlands, 1942), the question naturally arises as to the nature of the mechanism which ensures liberation of the enzyme at its point of action. The evidence presented in this paper enables certain conclusions to be drawn in this connexion. In the first place, the presence of hyaluronic acid, the substrate on which the enzyme acts, appears to have no effect in determining the liberation of the enzyme from spermatozoa, and it may be presumed that the presence of a cumulus is similarly without effect. Experiments designed to test the effect of saline extracts of Fallopian tube on the rate of disintegration of rabbit cumuli in sperm suspensions revealed a similar absence of effect (unpublished observation). No specific mechanism, therefore, which would ensure selective liberation of the enzyme in the Fallopian tube, appears to exist. The most significant light on this subject, however, would appear to be that afforded by observations on the effect of the concentration of sperm suspensions in Baker's solution on the quantity of hyaluronidase liberated. These indicate that as the sperms ascend the female reproductive tract, moving continually into a fresh medium, they undergo a progressive loss of hyaluronidase, the latter passing into solution in the fluid within the lumen of the tract. This being so, the concentration of hyaluronidase established in the Fallopian tube is actually lower than in other parts of the female reproductive tract.

The mode of release of hyaluronidase from spermatozoa has further implications. In the first place, it suggested that the presence of hyaluronidase in solution in the suspending medium would reduce the extent to which the enzyme was liberated by spermatozoa in such a suspension. This was, in fact, found to be the case and it throws an interesting light on the experiments of Rowlands (1944) on the capacity of hyaluronidase to increase the fertilizing power of dilute sperm suspensions in rabbits. In these experiments the sperm suspensions were made by diluting with Baker's solution for the controls, or with a hyaluronidase-containing sperm-free filtrate of a concentrated suspension of spermatozoa in Baker's solution, heated to 56° for a length of time just sufficient to kill the spermatozoa. The results showed that the treated inseminate required on the average one-sixth the number of spermatozoa as did the control inseminates, in order to fertilize a particular percentage of ova. The question naturally arose as to how the added hyaluronidase ascended the reproductive tract so as to assist fertilization. The unaided ascent of the enzyme was a possibility, but observations (unpublished) by the author on the passage through the reproductive tract of hyaluronidase introduced in solution into the vagina of rabbits suggest that though occasionally this may occur to a significant extent, in general it is not possible to demonstrate hyaluronidase activity in tubal washings of rabbits thus treated. The most probable explanation, therefore, is that the sperms in the treated inseminates retained a significantly greater proportion of their hyaluronidase which, accordingly, was available for action on the cumuli within the Fallopian tubes.

Recent observations by Chang (1946*a*, *b*) on the minimal numbers of sperms required to fertilize rabbit ova have revealed the existence of a variety of factors influencing such numbers. Thus, the percentage of ova fertilized by a given small number of sperms depends upon the number of ova ovulated and the maturity of the animal. More important from the point of view of present considerations is the finding that the dilution of an inseminate containing a small number of sperms affects its fertilizing capacity. Thus, the percentages of fertilized

ova were 17–42, 0–28 and 0–6% when inseminates containing 30,000–44,000 sperms had volumes of 0·1, 0·4 and 1·0 ml. respectively. In seeking an explanation for this effect, Chang (1946*b*) suggested that in the more dilute suspensions fewer sperms were able to enter the cervix uteri and so reach the Fallopian tubes. Though this may well be true it is probable that at least two other factors may apply. In the first place, the evidence presented in this paper shows that the sperms in the more dilute suspensions would contain less hyaluronidase, while in the second, it has been observed that the survival time of sperms in dilute suspensions in Baker's solution is greatly reduced. For example, whereas a suspension containing 10–20 millions/ml. may survive for 36 hr. or more at room temperature, the survival time of a suspension containing 1 million/ml. may be less than 1 hr. No explanation for this phenomenon has yet been found, but it has prevented the carrying out of an experiment which the previous results have obviously suggested, namely, the possibility of sperms whose hyaluronidase content has been depleted being made to reabsorb the enzyme on being suspended in a medium rich in the latter.

SUMMARY

1. Investigation of some of the factors concerned in the liberation of hyaluronidase from rabbit spermatozoa has shown that freezing for a short time or standing in aqueous suspension at 0° for 24 hr. or longer allows apparently maximal extraction of the enzyme. Freeze-drying may cause an insignificant loss of potency but the product remains stable for long periods.

2. At least three quarters of the total hyaluronidase content of semen is associated with the spermatozoa, the remainder being in solution in the plasma. In suspensions of live sperms in Baker's solution, the amount of hyaluronidase in solution is roughly proportional to the logarithm of the volume of suspension for a constant number of sperms. The presence of hyaluronidase in solution in the suspending medium reduces the amount of enzyme liberated by the sperms.

3. Spermatozoa do not appear actively to produce hyaluronidase; they merely liberate the preformed enzyme.

4. It is suggested that the mechanism which accounts for the relationship between volume of suspension and hyaluronidase liberated is sufficient to explain the release of enzyme in the Fallopian tubes necessary to bring about disintegration of the cumulus surrounding freshly ovulated ova.

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The Metabolic Conversion of Naphthalene to 1:2-Dihydronaphthalene-1:2-diol

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Studies of the metabolism of naphthalene (Bourne & Young, 1933*a*, *b*, 1934; Ing, Bourne & Young, 1934) and anthracene (Boyland & Levi, 1935, 1936*a*, *b*) have revealed certain similarities in the chemical changes which these hydrocarbons undergo in the animal body. Both are converted to mercapturic acids and both give rise to derivatives which yield the parent hydrocarbons when decomposed by acid. The purpose of the present work was to determine whether the administration of naphthalene to the rat is followed by the excretion of a derivative analogous to 1:2-dihydroxy-1:2-dihydroanthracene, a compound which was isolated by Boyland & Levi (1935) from the urine of rats and rabbits to whose diet anthracene had been added.

Naphthalene, in the form of a solution in corn oil, was injected subcutaneously into rats. From the

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urine excreted by the dosed animals it was possible to isolate a laevorotatory compound, the elementary analysis of which corresponded to $C_{10}H_{10}O_2$. A study of the structure of this compound led to the conclusion that it was 1:2-*dihydronaphthalene*-1:2-*diol*. Further work showed that this compound was present also in urine excreted by rats following the administration of naphthalene by stomach tube. Hitherto, 1:2-dihydronaphthalene-1:2-diol has not been shown to be a metabolite of naphthalene. Furthermore, this compound does not appear to have been described in the chemical literature.

EXPERIMENTAL

All the animals to which naphthalene was administered were male white rats. During the experimental period they were housed in cages which permitted the collection of urine separate from the faeces. The urine was collected daily and was stored in the refrigerator. The rats were fed on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Limited) supplemented with fresh milk daily and whole wheat bread twice a week. In order to avoid contamination of the urine with fallen food, the animals were fed in a separate cage twice daily for periods of 1 hr. each, and any urine excreted during these periods was not collected. The rats had access to water at all times.

Five experiments were conducted in each of which a group of 12 rats received by subcutaneous injection a total of 4·8 g. of naphthalene dissolved in corn oil. The naphthalene solution was prepared by dissolving 6 g. of the pure hydrocarbon in corn oil so as to give a solution the volume of which was 30 ml. Each rat was given an injection of 0·5 ml. of this solution (i.e. 0·1 g. of naphthalene) under the skin of the back on 4 successive days. The collection of urine was started immediately after the administration of the first dose of hydrocarbon and was continued until 2 days had elapsed after giving the fourth dose. The average weights of the rats in the various groups at the time of the first dosing with naphthalene were 416, 219, 414, 229 and 343 g.

Isolation of the naphthalene derivative from urine

The urine obtained in each experiment was treated separately. It was filtered through glass wool and its volume was measured. The urine volumes ranged from 530 to 720 ml. The urine was adjusted to pH 7–8 and it was then extracted with ether in a continuous extractor for a period of 9 hr. on each of 3 successive days. The ether extract obtained each day was evaporated to dryness under reduced pressure. Most of the material removed from the urine was extracted on the first day, a small amount was removed on the second day and almost none on the third day. The residues obtained by evaporation of the ether extracts were either oily or partly crystalline in nature, and they were dark brown in colour. They were combined and treated with 200 ml. of warm water until as much of the material as would dissolve had done so. The aqueous solution was cooled to room temperature and filtered. The material which did not dissolve in water contained most of the pigment. It was dissolved in 50 ml. of ether and the ether solution was shaken with four 25 ml. portions of water. These aqueous extracts were then combined with the main aqueous solution and extracted with ether in a continuous extractor for two periods of 9 hr. each. The ether extract, when evaporated to dryness under reduced pressure, yielded a light-brown crystalline residue. This material was extracted as completely as possible by refluxing with several 200 ml. portions of *cyclo*hexane. The *cyclo*hexane extracts were filtered while hot and were concentrated by evaporation. The precipitate which formed was separated by filtration and was crystallized from benzene. The material thus obtained was crystallized again from *cyclo*hexane and from benzene and in most cases yielded a colourless crystalline product, the melting-point of which was not raised by further crystallization.

In the five experiments in each of which a group of rats received a total of 4·8 g. of naphthalene, the amounts of the compound isolated were 0·270, 0·635, 0·499, 0·338 and 0·521 g., and they accounted for 4·5, 10·5, 8·2, 5·6 and 8·6% respectively of the naphthalene administered.

Properties of the compound isolated from urine

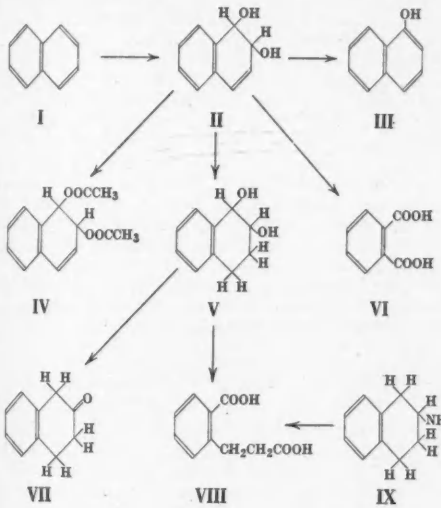
The compound separated from benzene in the form of colourless plates and from *cyclo*hexane as thin needle-shaped crystals. It melted with decomposition at 127–128° (this and other melting-points reported herein are uncorrected). $[\alpha]_D^{25°} = -154°$ (*c*, 1% in ethanol) and $[\alpha]_D^{24°} = -328°$ (*c*, 0·5% in water). Found: C, 74·1; H, 6·3%; mol. wt. 159. $C_{10}H_{10}O_2$ requires C, 74·0; H, 6·2%; mol. wt. 162.

In solid form the compound appeared to be stable under ordinary conditions, for a sample which was left at room temperature for 3 months showed no discoloration and no change in melting-point or specific rotation. At 23° the compound showed the following solubilities (in 100 ml. of solution): water, 2·26 g.; benzene, 0·27 g.; *cyclo*hexane, 0·01 g. It was very soluble in ethanol and in ether.

A solution (0·5%) in water gave no coloration when treated with dilute ferric chloride solution. No colour was produced when a solution of diazotized sulphanilic acid was added to a 0·25% solution of the compound containing an excess of NaOH. A strong red colour was produced when a 0·25% solution of the compound in N-HCl was boiled for 1 min., cooled, and treated with diazotized sulphanilic acid in the presence of excess NaOH. When a 0·25% solution of the compound in N-HCl was allowed to stand at room temperature and measured samples were withdrawn at intervals and submitted to the diazo test, the following reactions were observed: 1 hr., faintly positive; 4 hr., moderately positive; 24 hr., strongly positive. Colour development in the test just described was found (see below) to result from the coupling of diazotized sulphanilic acid with α-naphthol formed by acid decomposition of the compound from urine. Information on the stability of the compound in alkaline solution was obtained from an experiment in which 0·051 g. of the compound was dissolved in 5 ml. of 2N-NaOH solution and heated in a water-bath at 94–96° for 30 min. At the end of this period the solution was diluted with 10 ml. of water and extracted with four 20 ml. portions of ether. The ether extracts were evaporated to dryness and the residue was extracted with a small volume of dry ether. When evaporated to dryness, this ether extract yielded 0·048 g. of product, m.p. 126–127°, $[\alpha]_D^{25°} = -154°$ (*c*, 1% in ethanol).

Structure of the isolated compound

The conclusion that the compound isolated from the urine of rats dosed with naphthalene (I) was 1:2-*dihydronaphthalene*-1:2-*diol* (II) is based on the following evidence. The elementary analysis of the compound corresponded to $C_{10}H_{10}O_2$. The presence of a naphthalene ring structure with two hydroxyl groups, one of which was in position 1, was indicated by the conversion of the compound to a diacetate (IV) and by its decomposition to α-naphthol (III). The presence of both hydroxyl groups on the same ring was suggested by the finding that oxidation of the compound with potassium permanganate yielded *o*-phthalic acid (VI). These findings, in conjunction with the absence of phenolic properties and the fact that the compound was optically active, indicated that the compound was either 1:2-dihydronaphthalene-1:2-diol or 1:4-dihydronaphthalene-1:4-diol. On hydrogenation under suitable conditions, the compound took up two atoms of hydrogen to give a product $C_{10}H_{12}O_2$. This hydrogenated derivative was identified as 1:2:3:4-*tetrahydronaphthalene*-1:2-*diol* (V), which led to the conclusion that the compound isolated from urine was 1:2-dihydronaphthalene-1:2-diol.



The recognition of the hydrogenated derivative as 1:2:3:4-tetrahydronaphthalene-1:2-diol was based on the following findings. The analysis of the compound corresponded to $C_{10}H_{12}O_2$. A positive reaction was obtained when this compound was decomposed by acid and submitted to the test described by Straus & Rohrbacher (1921) for 2-keto-1:2:3:4-tetrahydronaphthalene (VII). On oxidation with potassium permanganate at 5–8° the hydrogenated compound yielded β-(2-carboxyphenyl)propionic acid (VIII). This acid was identified by comparison with the synthetic compound obtained by oxidation of 1:2:3:4-tetrahydro-2-naphthylamine (IX) prepared by reduction of 2-naphthylamine. The oxidation of 1:2:3:4-tetrahydronaphthalene-1:4-diol would not be expected to give rise to β-(2-carboxyphenyl)propionic acid. On the other hand, Straus & Rohrbacher (1921) have shown that this dicarboxylic acid is formed when the *trans* form of 1:2:3:4-tetrahydronaphthalene-1:2-diol is oxidized by potassium permanganate under conditions similar to those used in the present work to oxidize the hydrogenated derivative prepared from the compound isolated from urine.

Acid decomposition of the compound. A solution of 0·106 g. of the compound in 50 ml. of 2N-HCl was heated in a boiling water-bath for 15 min., cooled, and extracted with three 25 ml. portions of ether. The combined ether extracts were washed with 25 ml. of water, evaporated to dryness, and yielded 0·089 g. of residue. This residue dissolved almost completely when treated with 25 ml. of boiling light petroleum (b.p. 40–60°). The petroleum solution was filtered, concentrated and cooled. It gave a precipitate (0·070 g.), m.p. 95°, mixed m.p. with α-naphthol (m.p. 96° 95°. Found: C, 83·1; H, 5·8. Calc. for $C_{10}H_8O$: C, 83·3; H, 5·6%.

To 0·052 g. of the acid decomposition product were added 2 ml. of pyridine and 1 ml. of acetic anhydride. The solution which formed was refluxed gently for 15 min. and then poured into 10 ml. of water cooled in an ice-bath. The precipitate which separated was filtered and crystallized from aqueous methanol, giving 0·033 g. of product, m.p. 45°, mixed m.p. with α-naphthyl acetate (m.p. 45–46°) 45–46°. Found: C, 77·6; H, 5·5. Calc. for $C_{12}H_{10}O_2$: C, 77·4; H, 5·4%.

Acetylation of the compound. The compound (0·056 g.) was refluxed for 15 min. with 2 ml. of pyridine and 1 ml. of acetic anhydride. The solution was then poured into 10 ml. of water cooled in an ice-bath. The precipitate which formed was crystallized twice from aqueous methanol. The product was obtained as plates (0·052 g.), m.p. 78–79°, $[\alpha]_D^{25°} = -423°$ (*c*, 0·5% in ethanol). Found: C, 68·4; H, 5·8. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.

Oxidation of the compound. To a solution of 0·098 g. of the compound in 15 ml. of water were added 15 ml. of 4% $KMnO_4$ (aqueous). The mixture was heated in a boiling water-bath for 1 hr. and then cooled. SO_2 was bubbled through the mixture until all the precipitate dissolved, and the acid solution was then extracted with four 50 ml. portions of ether. The combined ether extracts were evaporated to dryness under reduced pressure and the residue thus obtained was crystallized from a small volume of water. The product was filtered and dried over P_2O_5 (0·055 g.). Neutralization equivalent, 84: calculated for *o*-phthalic acid, 83. Found: C, 57·9; H, 3·6. Calc. for $C_8H_6O_4$: C, 57·8; H, 3·6%.

When 0·021 g. of the oxidation product was heated at 130° under reduced pressure in a microsublimation apparatus, 0·017 g. of crystalline product was obtained, m.p. 129°. The mixed m.p. with phthalic anhydride was not depressed.

Hydrogenation of the compound. The compound was hydrogenated in glacial acetic acid solution in the presence of a platinum catalyst prepared by the method of Voorhees & Adams (1922). The catalyst (0·085 g.) was shaken in 15 ml. of glacial acetic acid in an atmosphere of hydrogen and took up 19·8 ml. of hydrogen in 45 min. 0·096 g. of the compound isolated from urine was then added and 15·3 ml. of hydrogen were absorbed in 6 min. at 759 mm. Hg pressure and 21°. The hydrogenation was then stopped. The theoretical uptake of hydrogen for the addition of two H atoms/molecule of compound was 14·3 ml. at the above temperature and pressure. The catalyst was separated by filtration, washed with glacial acetic acid, and the filtrate and washings were evaporated to dryness under reduced pressure. The residue was dissolved in methanol, the solution evaporated to dryness, and the residue then extracted with benzene. The benzene solution was filtered and when evaporated to a small volume it yielded 0·068 g. of crystalline product, m.p. 112–113°. This was dissolved in benzene, the solution filtered to remove traces of catalyst and then concentrated to small volume. This solution was cooled for a short time in the refrigerator, and the crystals which formed were filtered and washed with a little cold benzene, giving 0·050 g., m.p. 115°, $[\alpha]_D^{23°} = +62°$ (*c*, 0·5% in ethanol). Found: C, 73·2, H, 7·4. Calc. for $C_{10}H_{12}O_2$: C, 73·1, H, 7·4%. Two further hydrogenations were carried out in the manner just described. 0·100 g. of the compound isolated from urine was used in each hydrogenation, and together they yielded 0·116 g. of the hydrogenated derivative, m.p. 115°, $[\alpha]_D^{25°} = +61°$ (*c*, 0·5% in ethanol).

A solution of 0·010 g. of the hydrogenated derivative in 2 ml. of 2N-HCl was heated in a boiling water-bath for 15 min., and then cooled. A few drops of ethanol, 2 ml. of ether, and an excess of conc. NaOH were then added. When the mixture was shaken, the ether became a dark blue colour. According to Straus & Rohrbacher (1921), this test is given by 2-keto-1:2:3:4-tetrahydronaphthalene. A negative result was obtained when the hydrogenated derivative was subjected to the above test without first being decomposed by acid.

Oxidation of the hydrogenated derivative. The hydrogenated compound (0·083 g.) was moistened with 0·2 ml. of ethanol and then dissolved by the addition of 8 ml. of water. The solution was cooled to 5° in the refrigerator and 4·0 ml. of 4% $KMnO_4$ (aqueous) were added in 0·5 ml. portions over a period of 2 hr. while the temperature was maintained at 5–8°. The mixture was left overnight in the refrigerator and was then centrifuged. The precipitate was washed twice with water. The supernatant solution and the washings were combined, filtered, and extracted with three 50 ml. portions of ether. The ether extracts were evaporated to dryness and the residue was crystallized twice from benzene. 0·012 g. of crystalline material was obtained, consisting of unreacted hydrogenated derivative, m.p. 114°. The ether-extracted aqueous solution was made acid to Congo red by the addition of 2N-H_2SO_4, and then extracted with three 50 ml. portions of ether. The combined ether extracts were evaporated to dryness, treated with hot benzene, and the extract filtered. The filtrate was concentrated to small volume and the material which separated was crystallized from benzene (0·031 g.). When crystallized from water containing a little ethanol, it was obtained as long needles, m.p. 164–165° with preliminary sintering (alone, and when mixed with synthetic β-(2-carboxyphenyl)propionic acid (see below)). Found: C, 61·7; H, 5·1. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2%. Neutralization equivalent, 99; calc. for β-(2-carboxyphenyl)propionic acid, 97.

Synthesis of 1:2:3:4-tetrahydro-2-naphthylamine and its oxidation to β-(2-carboxyphenyl)propionic acid. In the course of their study of the structure of 1:2:3:4-tetrahydro-2-naphthylamine, Bamberger & Müller (1888) found that this compound yields β-(2-carboxyphenyl)propionic acid when oxidized. This reaction was used in the present work to prepare a sample of this acid for comparison with the product obtained by oxidation of the hydrogenated derivative of the compound isolated from urine.

1:2:3:4-Tetrahydro-2-naphthylamine was prepared by the reduction of 2-naphthylamine (10 g.) with sodium in boiling *iso*amyl alcohol by a method based on that of Gilman & Blatt (1941). The hydrogenated base was converted to its hydrochloride and most of the *iso*amyl alcohol removed by distillation. An excess of aqueous KOH solution was added to the residue, the mixture was extracted with ether, and 1:2:3:4-tetrahydro-2-naphthylamine carbonate (8·7 g.) was precipitated by the passage of CO_2 through the combined ether extracts. To 2 g. of this product in 100 ml. of water were added, with stirring, 50 ml. of 1% NaOH solution. While the temperature of the mixture was maintained at 8–12°, 125 ml. of 4% $KMnO_4$ (aqueous) were added in small portions over a period of 3 hr. The precipitate which formed was separated by centrifuging, washed with water, and the supernatant liquids were filtered. The filtrate was made acid to Congo red by the addition of 10 ml. of conc. HCl, and was extracted with four 200 ml. portions of ether. The ether extracts were combined and evaporated to dryness under reduced pressure. The residue was heated with 75 ml. of water, the solution was decanted from some oily material, and was then heated with charcoal and filtered. The filtrate was concentrated to a small volume and cooled in the refrigerator. The material which separated was filtered, washed with water, and dried (0·101 g.). It was crystallized first from benzene and then from dilute aqueous ethanol. 0·050 g. of long colourless needles was obtained, m.p. 164–165° with preliminary sintering. Found: C, 61·9; H, 5·1. Calc. for $C_{10}H_{10}O_4$: C, 61·8; H, 5·2%. Neutralization equivalent, 100; calc. for β-(2-carboxyphenyl)propionic acid, 97. Melting points recorded in the literature for this acid are 165–166° (Gabriel & Michael, 1877), 165·5° (Bamberger & Müller, 1888), and 165·5–167° (Straus & Rohrbacher, 1921).

Excretion of 1:2-dihydronaphthalene-1:2-diol by rats dosed with naphthalene by stomach tube

Twelve male white rats (average wt., 353 g.) were each given a suspension of 0·1 g. naphthalene in 1 ml. of 1% starch solution by stomach tube daily on 4 successive days. The other experimental conditions were similar to those in the experiments in which naphthalene was administered by subcutaneous injection. Treatment of the urine (vol., 500 ml.) by the method already described yielded 0·284 g. of crystalline material, $[\alpha]_D^{25°} = -153°$ (*c*, 1% in ethanol), m.p. 127–128°. The melting-point was not depressed when the compound was mixed with 1:2-dihydronaphthalene-1:2-diol isolated from the urine of rats dosed with naphthalene by subcutaneous injection. Found: C, 73·7; H, 6·2. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%. 0·071 g. of the compound was acetylated and yielded 0·073 g. of product,

$[\alpha]_D^{24°} = -430°$ (*c*, 0·5% in ethanol); m.p. 78–79°, the mixed m.p. with 1:2-dihydronaphthalene-1:2-diol diacetate (m.p. 78–79°) was unchanged. Found: C, 68·2; H, 5·8. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.

Other naphthalene derivatives excreted by rats dosed with naphthalene

From the urine of a group of 12 rats (average wt., 219 g.) which had been injected subcutaneously with naphthalene (total amount, 4·8 g.) under the conditions already described, 0·635 g. of 1:2-dihydronaphthalene-1:2-diol was isolated. The urine of these animals was then examined for the presence of *l*-1-naphthylmercapturic acid and a compound which yields naphthalene when decomposed by acid. These compounds have been shown to be present in the urine of rabbits dosed with naphthalene by stomach tube (Bourne & Young, 1933*a*, *b*, 1934) and in the urine of rats after the addition of naphthalene to their diet (Stekol, 1937; Chang & Young, 1943).

Half the urine (vol. 350 ml.) was made just acid to Congo red by the addition of conc. HCl, and 50 ml. of conc. HCl were then added. After being allowed to stand at room temperature for several hours, the acidified urine was extracted with four 400 ml. portions of chloroform. The chloroform extracts were combined, concentrated by evaporation, and extracted with 100 ml. of M-$NaHCO_3$ solution. The bicarbonate extract was made acid to Congo red by the addition of conc. HCl and left in the refrigerator overnight. The precipiate which formed was filtered, washed with water, and crystallized from aqueous ethanol. The pink crystals thus obtained weighed 0·653 g., m.p. 168–169°, mixed m.p. with *l*-1-naphthylmercapturic acid (m.p. 170–171°) 169–170°. The compound was purified further by precipitation as the sodium salt and reconversion to the acid by the method described by Bourne & Young (1934). When this material was crystallized from aqueous ethanol, 0·505 g. of *l*-1-naphthylmercapturic acid was obtained, $[\alpha]_D^{28°} = -25°$ (*c*, 1% in ethanol), m.p. and mixed m.p. 170–171°.

The remaining half of the urine was acidified to pH 2 by the addition of conc. HCl and allowed to stand overnight at room temperature. These conditions have been found to be optimal for the decomposition of the acid-decomposable naphthalene precursor excreted by rats following the ingestion of naphthalene (Chang & Young, 1943). The urine was then made alkaline to litmus and extracted with ether in a continuous extractor for 10 hr. The ether extract was washed with 2N-HCl, then with 2N-NaOH, and finally with water. It was then dried over anhydrous $CaCl_2$, filtered, and evaporated to dryness. The residue was extracted with hot light petroleum (b.p. 40–60°). The petroleum extract was filtered, evaporated to dryness in a current of air, and yielded 0·098 g. of crude naphthalene, m.p. 75–77°. This was purified by dissolving it in ethanol and adding water. The crystalline precipitate melted at 80° (alone and mixed with naphthalene).

Experiments similar to those just described were conducted with the urine of the 12 rats (average wt. 353 g.) which received a total of 4·8 g. of naphthalene by stomach tube. The urine of these animals yielded 0·284 g. of 1:2-dihydronaphthalene-1:2-diol. From half the urine (250 ml.), 0·431 g. of *l*-1-naphthylmercapturic acid was obtained, m.p. and mixed m.p. 169–170°. This material was precipitated as the sodium salt, reconverted to the acid, and when crystallized from aqueous ethanol 0·328 g. of the mercapturic acid was obtained, m.p. and mixed m.p. 170–171°, $[\alpha]_D^{28°} = -24°$ (*c*, 1% in ethanol). The other half of the urine yielded 0·151 g. of crude naphthalene, m.p. 76–78°, which when recrystallized from aqueous ethanol melted at 80° (alone and when mixed with naphthalene).

Amounts of compounds excreted

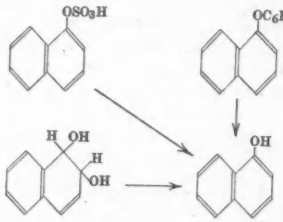
In the experiment in which naphthalene was administered by subcutaneous injection, 10·5% of the hydrocarbon was recovered from the urine as 1:2-dihydronaphthalene-1:2-diol, 12·1% as *l*-1-naphthylmercapturic acid, and 4·1% as naphthalene derived from the acid-decomposable precursor. When the naphthalene was given by stomach tube, 4·7% was obtained from the urine in the form of the diol, 8·0% as the mercapturic acid, and 6·3% as naphthalene liberated from the precursor. The amounts of hydrocarbon converted to these derivatives were certainly greater than the values just given, for the main purpose of the experiments was the separation of the compounds in a form suitable for identification, and some losses occurred during the purification processes.

DISCUSSION

The finding that naphthalene is converted to 1:2-dihydronaphthalene-1:2-diol in the rat provides the second example of a polycyclic hydrocarbon undergoing this type of metabolic change, for Boyland & Levi (1935) have shown that the corresponding anthracene derivative is excreted by rats and rabbits following the addition of anthracene to the diet. It seems probable that other polycyclic hydrocarbons give rise to derivatives of this type in the body. In preliminary experiments with the urine of rats dosed subcutaneously with solutions of phenanthrene in corn oil, the writer isolated small amounts of a crystalline product, m.p. 186–187°, the analysis of which corresponded to $C_{14}H_{12}O_2$. (Found: C, 78·9; H, 5·6. Calc. for $C_{14}H_{12}O_2$: C, 79·2; H, 5·7%.) This compound gave no colour when treated with diazotized sulphanilic acid in the presence of excess NaOH. When the compound was boiled with N-HCl for 1 min., cooled, and submitted to the diazo test, an orange-red colour was produced. This behaviour is similar to that shown by 1:2-dihydronaphthalene-1:2-diol, and when considered in conjunction with the analysis of the compound it suggests that in the rat phenanthrene may be converted to a dihydrophenanthrene-diol.

It has long been recognized that some cyclic hydrocarbons and certain of their derivatives are oxidized in the body to phenolic compounds which are excreted in the urine in the 'free' form or in conjugation with sulphuric acid or glucuronic acid.

Conjugated phenols are hydrolyzed when heated with strong acid, and heating the acidified urine is used commonly as a step in the isolation of phenolic compounds from urine. If the urine of rats which have been dosed with naphthalene is subjected to this treatment without previous removal of the 1:2-dihydronaphthalene-1:2-diol, this compound will be converted to α-naphthol. If this reaction is not known to have taken place, it might be supposed that this naphthol was originally present as such in the urine, or that it had been liberated by the hydrolysis of its sulphuric acid or glucuronic acid derivative:



A similar situation could arise with respect to the isolation of 1-anthrol following the acid hydrolysis of urine excreted by rats or rabbits which had been dosed with anthracene.

The fact that naphthalene and anthracene give rise to dihydroxy-dihydro derivatives which are decomposed by acid to yield monohydroxy compounds suggests that in studies of the metabolism of cyclic hydrocarbons consideration should be given to the possibility that part, at least, of a phenolic compound isolated from an acid-treated urine may have been formed by the breakdown of a dihydroxy-dihydro derivative of the hydrocarbon.

SUMMARY

1. A study of the structure of a laevorotatory compound isolated from the urine of rats dosed with naphthalene by subcutaneous injection or by stomach tube led to the conclusion that this compound was 1:2-*dihydronaphthalene*-1:2-*diol*. The amounts of this metabolite isolated from the urine accounted for 4·5–10·5% of the naphthalene administered.

2. The urine of rats dosed with naphthalene also contained *l*-1-naphthylmercapturic acid and a compound which yielded naphthalene when decomposed by acid.

3. The isolation of phenolic compounds after the acid hydrolysis of urine excreted by animals dosed with cyclic hydrocarbons is discussed in relation to the excretion of compounds of the type of 1:2-dihydronaphthalene-1:2-diol.

All the elementary micro-analyses reported above were carried out by Mr Michael Edson, who also gave valuable assistance in the conduct of the catalytic hydrogenations. A preliminary account of the work described in this paper was presented at a meeting of the Toronto Biochemical and Biophysical Society held on 25 April 1946 (Young, 1946).

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An Investigation into the Chemical Estimation of the Oestrogens in Urine

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In view of the great difficulties inherent in the biological estimation of the oestrogens, the introduction of a satisfactory chemical method would be a notable advance. Until recently no chemical method was claimed to be suitable for the estimation of the oestrogens in normal urine. The method of Kober (1931) and that of Bachman (1939) and others can be used only in the assay of pregnancy urine, of which the oestrogen content is nearly a thousandfold greater than that of normal urine. When, therefore, Talbot, Wolfe, MacLachlan, Karush & Butler (1940) introduced a new chemical method by which they claimed it was possible to estimate oestrone and oestradiol in normal urine, it was considered advisable to investigate the method, because the authors had not verified it by comparison with biological assay.

METHODS

The method of Talbot *et al.* consists essentially of the isolation of the weakly phenolic ketones in urine, coupling with diazotized dianisidine, and the measurement of the colour produced.

Extraction of urine. The method used is based upon that of Smith, Smith & Schiller (1939). The process initially used in the present series of investigations was carried out as described by Talbot *et al.* Details in the example of rat urine were as follows.

Urine was collected from a group of rats into a flask containing 1 ml. of conc. HCl and a sample taken, equivalent to the daily output of 10 rats (usually of the order of 70 ml., including rinsings). Conc. HCl (10 ml.) was added and the solution boiled under reflux for 10 min. After cooling it was extracted with ether (4 × 25 ml.). The ether extract was washed with 20% Na_2CO_3 (3 × 20 ml.) (all the coloured impurity is not removed by the single washing recommended by Talbot), and then extracted alternately with 0·1 N-NaOH (3 × 25 ml.) and 0·1 N-NaOH containing 10% $Na_2S_2O_4$ (2 × 25 ml.). (The extract was shaken with the hydrosulphite on each occasion for 3 min.) The aqueous residue containing strongly acidic and phenolic material (including oestriol) was discarded. The ether extract was washed with 0·5 N-HCl (2 × 25 ml.) and water (3 × 25 ml.), evaporated to dryness and redissolved in 50 ml. of toluene. This solution was extracted with N-NaOH (4 × 20 ml.) and the toluene residue, containing neutral ketosteroids, discarded. The alkaline extract was acidified to litmus but not to Congo red with conc. HCl and extracted with ether (5 × 30 ml.). The ether was washed alternately with 0·1 N-NaOH (2 × 20 ml.) and 0·1 N-NaOH-$Na_2S_2O_4$ (1 × 20 ml.), then with 0·5 N-HCl (2 × 20 ml.) and finally with water (3 × 25 ml.). The ether was evaporated and the residue taken up in 4 ml. ethanol and refluxed for 0·5 hr. with 0·5 ml. of glacial acetic acid and 0·5 g. Girard's reagent T. After the addition of 40 ml. of ice-water and 3 ml. of 10% NaOH, the non-ketonic fraction was extracted with ether (4 × 40 ml.). This ethereal extract contained the oestradiol and after washing with 0·1 N-NaOH (2 × 25 ml.) and water (2 × 25 ml.), was dried and ready for colorimetric assay.

The aqueous residue was acidified with 1 ml. of conc. H_2SO_4 and, after the addition of 60 ml. of water and 40 ml. of ether, left for 1·25 hr. at room temp. It was then extracted with ether (4 × 25 ml.) and the ether extract washed with 25 ml. of 0·1 N-NaOH and 3 × 25 ml. of water. This ether-soluble fraction contained the oestrone. The ether solutions were dried with anhydrous Na_2SO_4, the ether distilled off and 0·8 ml. of redistilled ethanol added. Two 0·3 ml. portions were used for the colour reaction.

It was found possible to shorten the extraction process as follows, without affecting the final assay figures: (1) The hydrosulphite washings were omitted. (2) The Girard reaction was completed in 15 min. instead of 30 min. (3) The Girard complex was hydrolyzed by boiling for 5 min., instead of standing at room temp. for 1·25 hr.

When the evidence (shown later) indicated that the shortened process gave results identical with those obtained by using Talbot's full extraction procedure, the shortened process was used for all the subsequent extractions.

The reagent was prepared and the colour reaction carried out as described by Talbot *et al.* The colour was estimated in a Spekker Photoelectric Absorptiometer, using a No. 6 blue filter (λ of max. transmission 4800–5000 A.).

Calibration curve. We had difficulty at first in constructing a calibration curve for oestrone, because we were unable to produce a consistent depth of colour with crystalline oestrone when the reaction was carried out as directed by Talbot *et al.* This difficulty was overcome by carrying out the reaction at about 0°. The ethanolic solution of the hormone containing the added Na_2CO_3 was immersed in ice and salt for a few minutes before addition of the reagent. Consistent results were then obtained.

As an example, four samples of urine extract were treated with diazotized dianisidine at room temperature and the absorptiometer readings were 0·320, 0·180, 0·185 and 0·145 respectively. When the reaction was carried out at 0° the readings were 0·135, 0·140, 0·135 and 0·138 respectively. The calibration curve using known amounts of oestrone (within the range of 5–50 μg.) was then very similar to that constructed from Talbot's data and identical with that for oestradiol.

RESULTS

Extraction process

A number of assays of urine was carried out using the full extraction method of Talbot and the modified procedure as described above. Both methods gave identical results as indicated in Table 1.

Table 1. *Comparison of Talbot's full extraction method and the modified procedure (rat urine)*

	Talbot method	Modified procedure
	(μg./10 rat-days)	
Ketonic fraction	27	29
Non-ketonic fraction	63	55

Assay of urine samples

Urine from three different sources, human male and female, and female rats (three batches), was extracted and the oestrogen estimated chemically. The results are given in Table 2.

Table 2. *Chemical estimation of oestrogens in urine*

	Ketonic	Non-ketonic
	(μg./100 ml.)	
Human urine (male)	38	73
Human urine (female)	93	77
	(μg./10 rat-days)	
Rat urine (female) 1	31	83
2	44	123
3	40	58

The values obtained for human urine are considerably higher than the levels usually accepted (male urine 5–15 μg./day, female 10–36 μg./day *total* oestrogen). We are unable to find figures in the literature for the oestrogen content of rat urine.

Comparison of biological and chemical assay

We decided to compare the results obtained by biological estimation of the oestrone in rat urine with those obtained by chemical estimation. The equivalent output of 200 rat-days of urine was extracted by the modified procedure and the ketonic fraction isolated. Three groups each of 10 mice were used for the urine assay and two control groups of 10 mice given crystalline oestrone. The mice were ovariectomized one week before injection. The material to be tested was dissolved in arachis oil and injected intramuscularly in three portions (0·25 ml. each) on the morning, evening and the following morning, and the vaginal smear examined on the 3rd, 4th and 5th day after the first injection. The results are shown in Table 3, from which it is evident that the biological estimation shows a content of 3 μg. of oestrone in the equivalent of approx. 360 rat-days of urine. This is in marked contrast with the chemical estimation of 3 μg. per rat per day.

It was clear that the chemical method measured material other than oestrogens. Evidence for this assertion was sought by carrying out procedures which could be expected to produce a marked change in the oestrogen content of urine.

Chemical assay of rat urine of varied oestrogen content

Urine from a group (A) of 14 rats was collected daily, before and after bilateral ovariectomy and the oestrogen content determined by the chemical method. It was anticipated that as a result of ovariectomy there would be a decreased output of oestrogen in the urine. The results in Table 4 indicate that there is no substantial difference in the oestrogen content of normal and ovariectomized rats' urine when assayed by the chemical method.

Table 4. *Chemical estimation of rat urine of varied oestrogen content*

	Ketonic	Non-ketonic
	(μg./10 rat-days)	
Before ovariectomy	30–35	60–100
2 days after ovariectomy	24	60
2 weeks after ovariectomy	26	114

It is well established that only 1–10 % of injected oestrogen can be recovered in the urine of women and experimental animals. The remainder cannot be traced in a biologically active form, and it is possible that it is excreted in the urine as a biologically inactive derivative of the oestrogens. We investigated the possibility that such an intermediate breakdown product may have been contributing to the colour reaction. Oestrone (150 μg.) was injected into each of a group (B) of 9 ovariectomized rats and a chemical assay of the urine excreted during the 48 hr. following the injection was carried out. Table 5 shows that the oestrone injection did not affect the excretion of material which reacts with diazotized dianisidine.

Eight duplicate estimations on the same batch of mixed human male urine gave values for the ketonic fraction ranging between 20 and 39, mean 30 μg./100 ml.; and for the non-ketonic fraction, 51 and 83,

Table 3. *Comparison of biological and chemical estimation of oestrogen content of rat urine*

Biological method	Control—cryst. oestrone	0·085 μg./mouse	7 animals in pro-oestrus
		0·135 μg./mouse	5 in oestrus: 5 in pro-oestrus
	Urine extract	16 rat-days/mouse	5 in oestrus: 4 in pro-oestrus. 1 death
Chemical method	Urine extract	16 rat-day sample	48 μg.

Table 5. *Chemical estimation of oestrogens in rat urine*

(Ovariectomized rats—group B—treated with 150 μg. oestrone.)

	Before injection (μg./10 rat-days)	After injection
Ketonic	24	20
Non-ketonic	57	60

mean 68 μg./100 ml. Thus it is to be expected that a 50% change in the oestrogen content of urine would be detectable by the chemical method. The administration of a total of 1350 μg. of oestrone to a group of rats would, therefore, be expected to result in a significant rise in the urinary content of colour-reacting material if this were due to a breakdown product of oestrone.

Diazotized dianisidine reacts with all phenolic groupings and the extremely high values obtained in these urine assays are probably due to non-oestrogenic phenolic compounds. This possibility was tested by removing the phenols from the urine.

Removal of phenols

The phenols were extracted from the urine according to the method of Schmulovitz & Wylie (1935–6). In this method, the volatile phenols (phenol and cresol) are removed by steam volatilization. The non-volatile phenols (pyrocatechol) and the aromatic hydroxy acids (*p*-hydroxybenzoic, phenylacetic and phenylpropionic acids) are removed by exhaustive extraction from ethereal solution by sodium carbonate, while oestrone and oestradiol are not affected. This stage was included in the extraction of a sample of mixed male urine prior to the first ether extraction.

The ketonic fraction of the urine according to the chemical estimation was 500 μg. in a 24 hr. sample before phenol removal and 42 μg. in a 24 hr. sample after treatment. This last figure is approximately within the range of the accepted values obtained by biological methods. (The non-ketonic—oestradiol—fraction was too strongly coloured after this extraction procedure to be estimated colorimetrically.)

The excessively high values obtained by Talbot's method thus appear to be due to the incidental assay of phenols other than the oestrogens.

Recovery of added oestrone

A sample of mixed human male urine was extracted and after removing the phenols was assayed in duplicate. A portion of this urine was assayed after the addition of 200 μg. of oestrone. None of the added hormone was detected.

Hence despite this modification of the process the chemical estimation of oestrogens in urine still remains unsatisfactory.

DISCUSSION

The results reported in the present paper show that Talbot's method for the chemical estimation of the oestrogens in urine gives results many times higher than those obtained by the biological method. If the chemical method gave a true measure of the oestrogen content of urine, the values obtained for the urine of totally ovariectomized animals should be considerably lower than in the case of normal animals. Such results were not obtained. This indicates that it is not oestrogenic material which is producing colour reaction.

The theory that the colour developed might be due to a biologically inactive metabolic product of oestrogen became untenable as the result of injecting a large dose of oestrone into a group of rats. If any appreciable proportion of the large dose of oestrone had been thus excreted it would have been readily detected, as the method has been shown to be capable of demonstrating a 50% change in the urine content of material which reacts with diazotized dianisidine.

When the phenols are removed the results of the chemical estimation of the oestrogens are of the same order as those obtained by the biological method. Thus Talbot's method, relying as it does upon the isolation, by means of Girard's reagent, of weakly phenolic ketones (of which oestrone is said to be the only example present in urine) does not in fact effect this isolation and estimates other phenolic compounds.

It is clear that no chemical method is yet available for the estimation of oestrogens in normal urine and the biological assay remains the only reliable method.

SUMMARY

An investigation of Talbot's method (Talbot *et al.* 1940) of estimating oestrogenic substances in urine indicates that the results so obtained do not represent quantitatively the amount of oestrogen excreted in the urine.

We are indebted to Dr C. G. Paine for his valuable co-operation in carrying out the biological assays. The greater part of the expenses of this work were defrayed by a grant from the Nuffield Provincial Hospitals Trust and the remainder by a grant to one of us (A. W.) from the Medical Research Council.

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The Tryptophanase-tryptophan Reaction

8. THE MODE OF FORMATION OF INDOLE

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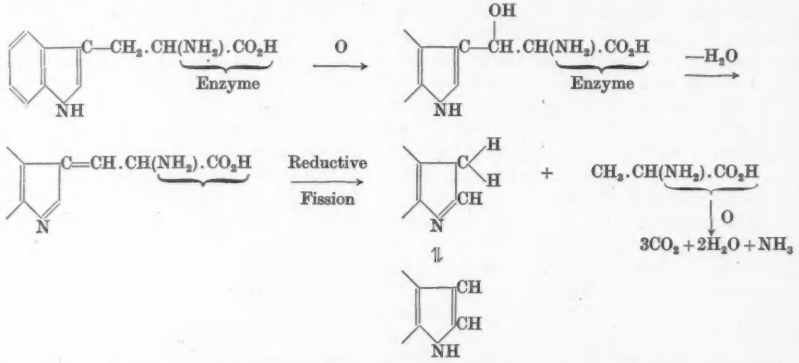
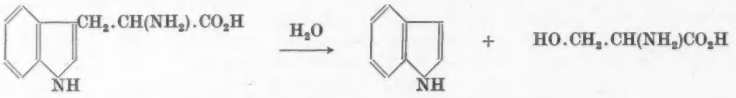
The work of Woods (1935) and of Happold & Hoyle (1935) had failed to throw light on the intermediate steps in the production by *Escherichia coli* of indole from tryptophan. Baker & Happold (1940) examined a number of tryptophan intermediates in which the alanine side chain had been systematically modified. Their results showed that the breakdown to indole required, *inter alia*, (*a*) a free carboxyl group, (*b*) an unsubstituted amino group and (*c*) a β carbon atom open to oxidative attack. A tentative mechanism for the breakdown of tryptophan to indole by *Esch. coli* was suggested, involving the production of alanine according to the following scheme:



Bonner & Tatum (1944), from a study of the way in which a mutant strain of *Neurospora crassa* synthesized tryptophan from indole and serine, postulated that the breakdown of tryptophan by *Esch. coli* was a reversal of this reaction, i.e. hydrolytic fission to yield indole and serine:



In support of this they quoted experiments in which inhibition of indole production was obtained by the addition of serine to tryptophan, and also experiments in which the synthesis of tryptophan by *Esch. coli* from indole and serine was claimed.

The present communication describes further experiments on the mechanism of the reaction and to test the validity of the above hypotheses (see also Dawes, Dawson & Happold, 1946). The effect of serine and alanine upon indole production has been tested and growth experiments performed. The detection of an intermediate or intermediates produced in the reaction is of crucial importance and since both the above mechanisms involve the fission of an intact side-chain, in each case an amino-acid, the method of partition chromatography on filter paper of Consden, Gordon & Martin (1944) was used. An inherent difficulty in much work of this kind is that the intermediate is immediately metabolized and so never reaches an appreciable concentration. This difficulty was somewhat alleviated by the observation of Dawson (1946) that under the influence of mepacrine there was no oxygen uptake over an initial 60 min. whereas indole was formed in diminished quantity. Hence, presumably, fission occurs giving indole and intermediate but the latter cannot be metabolized. There is however the possibility that metabolism of the intermediate might occur through internal hydrogen donation.

METHODS

Washed suspensions of *Esch. coli* were obtained from 18 hr. growth at 37° on tryptic digest of casein solidified with agar. Indole was determined by the method of Happold & Hoyle (1934). For growth experiments involving indole utilization, a modified Fränkel's medium was used, viz.: 5 g. NaCl, 4 g. asparagine, 6 g. ammonium lactate, 2 g. KH_2PO_4, 0·4 mg. $FeSO_4.7H_2O$, and 4 ml. mineral salt solution; made up to 1 l. with distilled water. The mineral salt solution contained in 50 ml. distilled water: 500μg. $CuSO_4.5H_2O$; 400μg. $ZnSO_4.7H_2O$; 200μg. $MnCl_2.4H_2O$; 0·35 g. $MgSO_4.7H_2O$. The method of analysis employed was partition chromatography on filter paper (Consden *et al.* 1944). Whatman's no. 1 filter paper was originally used but changed to no. 4 on the advice of Dr Martin.

RESULTS

Effect of serine on indole production

Two series of flasks were set up with contents:

(*a*) 20 ml. phosphate buffer pH 7·4 + 1 mg. *dl*-tryptophan.

(*b*) 20 ml. phosphate buffer pH 7·4 + 1 mg. *dl*-tryptophan + 0·25 mg. *dl*-serine.

These were incubated at 37° for 30 min. and then 0·5 ml. washed *Esch. coli* suspension added and re-incubated. Indole was determined at 15 min. intervals. The effect of the addition of equimolecular proportions of *dl*-serine compared with *dl*-tryptophan is shown in Fig. 1. Marked inhibition of indole production was obtained.

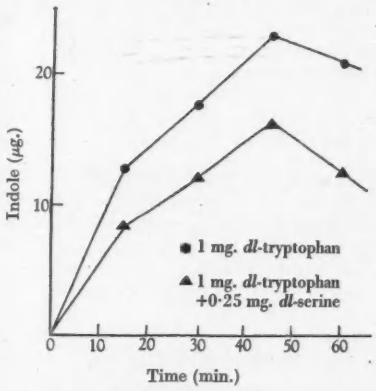


Fig. 1. Effect of serine on indole production by suspensions of *Esch. coli*.

Experiments were also performed using 0·5 mg. *dl*-serine/flask, i.e. double the molecular equivalent of *l*-tryptophan, but in various experiments, using different bacterial suspensions, Bonner & Tatum's (1944) observation that the degree of inhibition was directly proportional to the serine concentration was not confirmed; we found occasionally that, using different bacterial suspensions, a greater inhibitory effect was produced by equimolecular concentrations of serine than by double that amount. This appeared to be a function of the enzyme concentration; further experiments to test this in direct comparison with the effect of alanine are given below.

Effect of alanine on indole production

Two series of flasks were set up:

(*a*) 20 ml. phosphate buffer pH 7·4 + 0·5 mg. *dl*-tryptophan.

(*b*) 20 ml. phosphate buffer pH 7·4 + 0·5 mg. *dl*-tryptophan + 0·25 mg. *dl*-alanine.

As in the case of serine, each flask had 0·5 ml. bacterial suspension added. Fig. 2 shows the effect of alanine to be negligible, indole production being parallel with that of the control flask; but here again in certain experiments a degree of inhibition was detected.

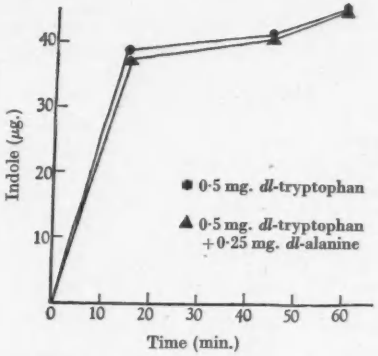


Fig. 2. Effect of alanine on indole production by suspensions of *Esch. coli*.

Effect of serine and alanine on indole production at varying enzyme concentration

To investigate the effect of enzyme concentration, parallel experiments on indole production in the presence of serine and alanine were done using four different concentrations of bacterial suspension, e.g. x, $0{\cdot}75x$, $0{\cdot}5x$, $0{\cdot}25x$.

Flasks were set up in three series:

(*a*) 20 ml. phosphate buffer pH 7·4 + 0·5 mg. *l*-tryptophan.

(*b*) 20 ml. phosphate buffer pH 7·4 + 0·5 mg. *l*-tryptophan + 0·5 mg. *dl*-alanine.

(*c*) 20 ml. phosphate buffer pH 7·4 + 0·5 mg. *l*-tryptophan + 0·5 mg. *dl*-serine.

Esch. coli suspension (0·5 ml.) was added to each flask and indole determined at 10 and 20 min. The results of two such experiments are given in Tables 1 and 2.

Table 1. *Effect of serine and alanine on indole production by* Esch. coli *suspensions*

(T = *l*-tryptophan, 0·5 mg.; S = *dl*-serine, 0·5 mg.; A = *dl*-alanine, 0·5 mg.)

Bacterial dry weight (mg.):	21·0			15·75			10·5			5·25		
Amino-acids present:	$T+A$	$T+S$	T	$T+A$	$T+S$	T	$T+A$	$T+S$	T	$T+A$	$T+S$	T
Time of contact (min.)	Indole produced (μg.); theory, 285 μg.											
10	200	198	210	207	215	218	—	—	—	80	98	111
20	252	234	248	247	247	255	284	268	272	254	215	241
	Indole produced (μg./mg. dry weight of bacteria)											
10	9·5	9·4	10·0	13·1	13·7	13·8	—	—	—	15·2	18·7	21·0
20	12·0	11·1	11·8	15·7	15·7	16·2	27·0	25·0	26·0	48·8	41·0	46·0

There was a possibility of some loss of indole by volatilization during the experiments.

In the case of alanine, retardation of indole production only occurred at the lowest enzyme concentration after 10 min.; at other concentrations, after 10 min. there was no significant retardation, while there was none at all after 20 min. with any concentration.

With serine, inhibition of indole production was manifest at 10 and 20 min. with the lowest enzyme concentration but was not significant at other concentrations where the tryptophanase was not saturated with tryptophan (Table 2). In this experiment, after 10 min., inhibition was produced by both alanine and serine to a slight degree at 24 mg. dry-bacterial weight, more so at 16 mg. and markedly at 8 mg. After 20 min., only in the weakest enzyme system was retardation produced by both alanine and serine. Thus the inhibition appeared to be marked only as saturation of tryptophanase with tryptophan was reached. At high enzyme concentrations, there was no inhibition of indole production by serine.

Table 2. *Effect of serine and alanine on indole production by* Esch. coli *suspensions*

(T = *l*-tryptophan, 0·5 mg.; S = *dl*-serine, 0·5 mg.; A = *dl*-alanine, 0·5 mg.)

Dry bacterial weight (mg.):	32			24			16			8		
Amino-acids present:	$T+A$	$T+S$	T	$T+A$	$T+S$	T	$T+A$	$T+S$	T	$T+A$	$T+S$	T
Time of contact (min.)	Indole produced (μg.)											
10	225	268	226	213	209	226	143	143	226	118	120	146
20	233	191	220	216	223	219	206	199	196	199	194	218
	Indole produced (μg./mg. dry weight of bacteria)											
10	7·0	8·4	7·0	8·8	8·7	9·4	9·0	9·0	10·5	14·8	15·0	18·2
20	7·3	6·0	6·9	9·0	9·3	9·1	12·8	12·2	12·1	25·0	24·2	27·2

Indole utilization by Esch. coli

The utilization of indole by *Esch. coli* in growth experiments over 7-day periods was demonstrated by Happold & Hoyle (1936). In the light of Bonner & Tatum's (1944) work on indole utilization in the presence of serine, these experiments were repeated using serine.

Erlenmeyer flasks were set up in three series with contents:

(*a*) and (*b*) 100 ml. Fränkel's medium + 0·002 g. indole.

(*c*) 100 ml. Fränkel's medium + 0·002 g. indole + 0·002 g. *dl*-serine.

After autoclaving, flasks of series (*b*) and (*c*) were inoculated with a loopful of light *Esch. coli* suspension and then incubated; (*a*) constituted the uninoculated control. Flasks were originally left for 24 hr. but as no utilization was recorded this was increased to 7 days. Indole was then determined in

Table 3. *Indole utilization by cultures of* Esch. coli *in the presence of serine*

Time (days)	Indole present (μg./10 ml. modified Fränkel's medium) (*a*)		(*b*)		(*c*)	
0	200	200	200	200	200	200
7	76·9	63·6	64·9	66·5	74·6	77·5

(*a*) 200 μg. indole/10 ml. uninoculated.
(*b*) 200 μg. indole/10 ml. inoculated.
(*c*) 200 μg. indole + 200 μg. *dl*-serine/10 ml. inoculated.

There was considerable loss of indole due to volatilization.

10 ml. samples taken from each flask. The results are given in Table 3, from which it will be seen that there was no utilization of indole in the presence of serine in excess of that in the inoculated flasks containing indole only.

Analytical investigation

Since oxygen uptake under the influence of mepacrine began after 60 min., the method adopted was to incubate the reaction flasks for 55 min. and then to work up the contents for analysis. Five flasks were set up with contents: 3·4 ml. phosphate buffer pH 7·4 + 0·8 ml. 1% (w/v) *dl*-tryptophan solution + 0·5 ml. 1% (w/v) mepacrine hydrochloride solusion + 0·3 ml. *Esch. coli* suspension. The flasks were incubated for 55 min. and then the enzyme system inactivated by shaking with ether. The contents of the flasks were pooled, the ether distilled off and the residue concentrated to approximately 1 ml. when some unchanged *d*-tryptophan separated from solution. This was removed by centrifugation and the supernatant used for analysis alongside control mixtures of tryptophan + serine and tryptophan + alanine. The solvent used was a solution of *p*-cresol saturated with water and chromatograms were run for 12–18 hr. The first few experiments gave no evidence of the presence of serine, but a faint coloration with ninhydrin was given in a position which approximated to the R_F value of alanine and which corresponded to the alanine of the control solution. On changing to *n*-butanol—benzyl alcohol saturated with water, alanine was confirmed.

Since the presence of mineral salts has a detrimental effect upon these analyses, a modification was later introduced. The contents of the flasks were shaken up with 'Deminrolit' but it was found that this removed amino-acids in addition to salts and so the method was discarded. Another method tried was the addition of the calculated amount of mercuric sulphate in sulphuric acid to remove unchanged tryptophan. The precipitate was collected on the centrifuge and the supernatant tested for further precipitation. Excess Hg^{++} was removed with hydrogen sulphide and excess SO_4^{--} with barium hydroxide. Finally the phosphate buffer of the medium was replaced by distilled water pH 6·4 and maximum indole production was thereby prolonged to approximateiy 3 hr. on account of the acid environment. Accordingly the flasks were incubated for 2·5 hr. before concentrating for analysis.

With the salt effect thus eliminated, better results were obtained and with three different solvents the presence of alanine was confirmed.

In later experiments, further modification of the contents of the reaction flasks was introduced. One flask was set up with contents: 20 mg. *dl*-tryptophan + 200 mg. mepacrine hydrochloride + 5 ml. *Esch. coli* suspension; made up to 200 ml. with distilled water. Improved results were obtained by this technique. In all the analyses performed serine was never detected. Results obtained using different solvents are summarized in Table 4.

Table 4. *Summary of analyses for alanine and serine, using partition chromatography, of products of reaction between washed suspensions of* Esch. coli *and tryptophan in the presence of mepacrine*

Solvent (saturated with water)	Alanine present (experiments)	No indication of alanine (experiments)
p-cresol	5	3
n-butanol-benzyl alcohol	8	2
n-butanol	1	1

Serine was always absent.

Oxygen utilization in presence of alanine, tryptophan and serine

Barcroft respirometers were set up with contents: 4·7 ml. phosphate buffer pH 7·4 + 0·3 ml. *Esch. coli* suspension to which amino-acids were added as follows: (*a*) nil, (*b*) 250μg. *l*-tryptophan, (*c*) 125μg. *dl*-alanine, (*d*) 125μg. *dl*-serine. The oxygen utilization of *Esch. coli* on these substrates is given in Table 5. Both alanine and serine were oxidized more rapidly than tryptophan, the serine initially at a faster rate than the alanine. This was not true of many other amino-acids, including glycine, histidine and phenylalanine, which were studied similarly.

Table 5. *Oxygen utilization by washed suspensions of* Esch. coli *with serine, alanine and tryptophan as substrates*

Time (min.)	*l*-tryptophan 250μg.	*dl*-alanine 125μg.	*dl*-serine 125μg.
	O_2 uptake (μl.)		
5	5·85	8·81	5·81
10	15·0	21·0	26·2
15	25·4	22·7	36·5
20	32·7	54·9	40·9
30	41·9	62·7	41·0
40	46·7	62·1	—

DISCUSSION

The evidence of Bonner & Tatum (1944) in support of their serine hypothesis is twofold. Serine exerts an inhibitory effect upon indole production from tryptophan by *Esch. coli*, this inhibition being directly proportional to the serine concentration and a mass action effect is suggested. Our experiments, while confirming this inhibitory effect have shown that the effect of serine is not a simple relationship between tryptophan and serine concentrations but rather of saturation of tryptophanase with tryptophan. The degree of inhibition produced by serine in concentrations of 1 or 2 equiv. in relation to the

tryptophan present is a function of the enzyme concentration and it would appear that the mass-action effect may only be manifest when the tryptophanase is fully saturated with tryptophan. When this condition is not fulfilled, serine does not appear to affect indole production and in some cases may stimulate it. Experiments in which alanine was added to tryptophan have been performed, and also parallel investigations using different enzyme concentrations. Here, as with serine, it was found that when the enzyme was not fully saturated with tryptophan there was no inhibition at all, but when saturation was being approached there was some inhibition, though it was usually less well marked than with serine. Thus the results obtained on the effect of alanine and cited above were cases where saturation of enzyme with tryptophan had not been achieved.

It is possible to explain these results in the manner of Bonner & Tatum but equally, since indole formation is dependent upon the continuing reoxidation of the carrier system of the tryptophanase complex (indole can be produced in anaerobic culture by electron transfer) and since alanine and serine are preferentially oxidized as compared with tryptophan by tryptophan-adapted cells, such inhibition may be explained by an insufficiency of oxygen to complete the reoxidation of the carrier system for both sets of reactions, viz. (*a*) the initial fission of alanine from the indole nucleus and (*b*) the oxidation of the amino-acid side chain after fission in the presence of the added alanine or serine.

Secondly, Bonner & Tatum quote experiments in which indole and serine were incubated with *Esch. coli* suspensions. In their own words 'Colorimetric tests for tryptophan after 24 hr. incubation indicated that some tryptophan had been synthesized from indole in the presence of serine', but experimental details and quantitative results are not given. All attempts by us to demonstrate indole utilization in the presence of serine in growth experiments over 7-day periods have failed. While this work was in progress Fildes (1945), using suspensions of *Esch. coli* and *Eberthella typhosa*, reported failure to demonstrate the utilization of indole in the presence of serine or production of tryptophan.

A study of the oxygen uptake of *Esch. coli* on alanine, serine and tryptophan substrates has shown that both alanine and serine are oxidized at a greater rate than tryptophan. This possibly explains why there is no real mass-action effect upon indole production when alanine or serine is added to tryptophan. Studies of oxygen uptakes on other amino-acids including glycine, histidine and phenylalanine showed that this was not true of these compounds.

The crucial evidence to decide in favour of one or the other of the two hypotheses must come, however, from definite detection of that particular intermediate. The difficulty here is that the breakdown of tryptophan to indole is complete within approximately 45 min., and any intermediate formed is immediately metabolized to carbon dioxide, ammonia and water. By the use of mepacrine as inhibitor, the production of indole still occurs, but in diminished amount, while there is no oxygen uptake over the initial 60 min. A detectable concentration of any intermediates might be built up under these conditions. With this technique we have analyzed solutions after 55 min. incubation with *Esch. coli*, and alanine has been detected in several such experiments whereas serine has never been found. The partition chromatography method of Consden *et al.* (1944) has enabled us to detect the alanine intermediate and the density of the colour reaction developed with ninhydrin has been consistent with the concentration expected from the concurrent liberation of the amount of indole determined in the majority of experiments performed.

Since in each experiment a control mixture of tryptophan and alanine (or serine) was used alongside the solution under investigation, our results were obtained by direct comparison and are not entirely dependent upon calculation of the R_F value, which may be affected by salt concentration and other variables. Nevertheless, in all cases we obtained good agreement with the accepted R_F values for alanine in the solvents employed. Thus the fact that the relative position of the unknown intermediate and *d*-tryptophan is paralleled by alanine and tryptophan in control runs, using three different solvents, places beyond doubt the production of alanine in the tryptophanase-mepacrine-indole system, and consequently by analogy in the tryptophanase-indole system itself.

The criticism usually levelled at the detection of intermediates in an inhibited reaction, namely that the normal reaction course is blocked, giving rise to unusual products, does not hold since mepacrine only slows down the reaction rate. In the presence of mepacrine, indole formation goes to completion, the overall oxygen utilization is the same as for tryptophan alone, but the reaction takes twice as long to reach completion. It is the metabolism of the side chain which is retarded. It is desirable that alanine should be detected in the absence of mepacrine, but all experiments conducted with this object have failed, on account of its rapid oxidation.

These investigations support the suggested hypothesis of Baker & Happold (1940) and alanine has been demonstrated conclusively as an intermediate produced in the breakdown of tryptophan to indole by *Esch. coli*. This does not constitute a criticism of Bonner & Tatum's work and views on *Neurospora*, but only of their application to the tryptophanase system of *Esch. coli*.

SUMMARY

1. Serine and alanine have an inhibitory effect upon indole production from tryptophan by *Esch. coli*. This effect is not a simple relation between the tryptophan and serine concentrations, and only holds when the enzyme is saturated with tryptophan.

2. Fildes's (1945) finding that there is no evidence of tryptophan synthesis from indole and serine using cells of *Esch. coli* is confirmed.

3. Using partition chromatographic analysis and retarding the reaction with mepacrine, alanine has been detected as an intermediate; under similar conditions the presence of serine has never been detected.

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Studies on Alcaptonuria

1. THE ESTIMATION OF HOMOGENTISIC ACID

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An accurate and specific method for the estimation of homogentisic acid applicable to small amounts of urine and plasma was required in connexion with experiments which are described in subsequent papers (Neuberger, Rimington & Wilson, 1947; Neuberger & Webster, 1947). Since it was intended to feed substances closely related to homogentisic acid, it was important to define clearly the specificity of the methods used. The present paper deals firstly with methods already described in the literature, and secondly with a new method which, it is believed, is both very specific and sensitive for homogentisic acid.

Baumann (1892), who established the structure of homogentisic acid, also described a method for its estimation in the urine, based on the reduction of silver nitrate in the presence of ammonia. The method is not specific and it is known that normal constituents of urine such as uric acid also reduce silver ions in an alkaline medium (Garrod & Hurtley, 1905). But in spite of this lack of specificity the many important results on human alcaptonuria obtained by the method of Baumann can be considered substantially correct, for the concentration of homogentisic acid in the urine of alcaptonurics is very high compared with that of other substances having similar reducing properties. For our purpose, however, the method was unsuitable and we have not investigated it further.

A more convenient method which makes use of the reduction of phosphomolybdic acid by hydroquinone and its derivatives in acid solution has been described by Briggs (1922). This method, which has been widely used in America, has considerable disadvantages if other substances arising from the metabolism of aromatic amino-acids are present. Thus Medes (1932) found that both *p*-hydroxyphenylpyruvic acid and 3:4-dihydroxyphenylalanine reduce the phosphomolybdate reagent in an acid medium, though the latter only very slowly. A similar experience was reported by Sealock & Silberstein (1940) who tried to differentiate between the two acids by reading the colour 5 min. after the addition of the final reagent, making allowance for the amount of colour produced in that period by the keto acid. Since similar difficulties were anticipated in our work, we have studied the rate of development of the colour with different concentrations of homogentisic acid in some detail, and have also investigated the validity of Beer's law and the specificity of this reaction.

Another method for the estimation of homogentisic acid is based on an observation by Wieland (1910) who found that hydroquinone is quantitatively oxidized to *p*-quinone by iodine in the presence of sodium bicarbonate without any substitution taking place in the benzene ring. On acidification the quinone reacts with the iodide ion, and is itself reduced again to hydroquinone and a corresponding amount of iodine is liberated which can be titrated with

thiosulphate. That homogentisic acid behaves in an identical manner was shown by Metz (1927). Conditions for the estimation of this acid in the urine were worked out (Lieb & Lanyar, 1929; Metz, 1930) and it was shown that homogentisic acid added to normal urine can be recovered to the extent of 97–98%. As might be expected, the consumption of iodine in slightly alkaline solution is not a specific reaction, since many constituents of normal urine are oxidized under such conditions. The liberation of iodine on acidification, however, should be peculiar to substances having oxidation-reduction potentials of the same order of magnitude as homogentisic acid and showing a similar variation with pH. It was shown by Blix (1932) that this reversible reaction of hydroquinone and homogentisic acid with iodine is a consequence of the variability of the oxidation-reduction potential of the hydroquinone-quinone system with pH, whilst the corresponding potential of the iodine-iodide system is independent of acidity changes. Blix has shown that it is necessary to reduce the pH to about 0, in order to ensure that the reaction goes practically to completion, i.e. to about 99·5%.

But apart from the equilibria involved, there are other factors to be considered. The reactions, both oxidation and reduction, are not instantaneous and their respective rates should vary with the pH. Moreover, the quinone is not very stable in alkaline solution and the possibility of a substitution by iodine has also to be considered. Our first aim was therefore to establish the most favourable conditions for a quantitative recovery of homogentisic acid. It also appeared possible that substances containing amino or thiol groups might react with the quinone and thus interfere with the estimation. Finally we wished to investigate whether this reaction also occurs with 1:2-dihydroxybenzenes, such as dopa and catechol.

During the course of this work it was found that, though the iodometric method was more specific than that of Briggs, several substances which might either occur normally in urine or be formed in our feeding experiments behaved towards iodine exactly like homogentisic acid, whilst others interfered to some extent with the reaction. Attention was therefore directed towards finding an even more specific method of estimation and it was proposed to take advantage of the relative solubilities of homogentisic acid in different solvents and of the fact that it reduces silver ions in acid solution. It was proposed therefore, to extract the homogentisic acid first from water into ether and then back again into water and then estimate it by determining its reducing power at a well-defined pH. If homogentisic acid is added to a silver nitrate solution in the presence of acetate buffers between pH 4–5, colloidal silver is slowly precipitated, but this precipitate cannot be readily estimated by turbidimetry. In the presence of a protective colloid such as gum arabic a silver sol is formed, but it was found that the colour and therefore presumably the particle size varied in an uncontrollable manner. This agrees with the work of Voigt & Heumann (1928) who noted that silver sols rarely obeyed Beer's law. These authors also found that if the reduction of the silver salt is carried out in the presence of colloidal gold of uniform particle size, fairly homogeneous silver sols are obtained. We have used this observation and have added a minute amount of gold sol to the buffered mixture before the addition of homogentisic acid. The most satisfactory results were obtained when the gold sol was prepared by the old method of Faraday (1857).

PART 1. BRIGGS'S METHOD

Experimental

Reagents. The composition of the reagents used was the same as that given by Briggs (1922), viz. a phosphate solution containing 1% KH_2PO_4 (A.R.) and a molybdate solution containing 5% ammonium molybdate (A.R.) in 5N-H_2SO_4. The standard was a solution of 0·1% homogentisic acid which was made up fresh every second day and kept at 0°. The homogentisic acid used was a synthetic sample thrice recrystallized from an ether-chloroform mixture.

Procedure. A known volume of the solution to be estimated was diluted to about 15 ml. with water. To this was added 2 ml. of molybdate solution and 2 ml. of the phosphate solution. The volume was then made up with water to 25 ml. Readings were taken after exactly 1 hr. unless otherwise stated with a Hilger absorptiometer with a red filter.

RESULTS

Development of the colour. The maximum colour is not produced at once at any concentration tested and the rate of colour development varies with the concentration of homogentisic acid (Fig. 1). At the lower concentration the rate of change after 1 hr. is only small and this period was therefore allowed in all later experiments for the colour to develop. At

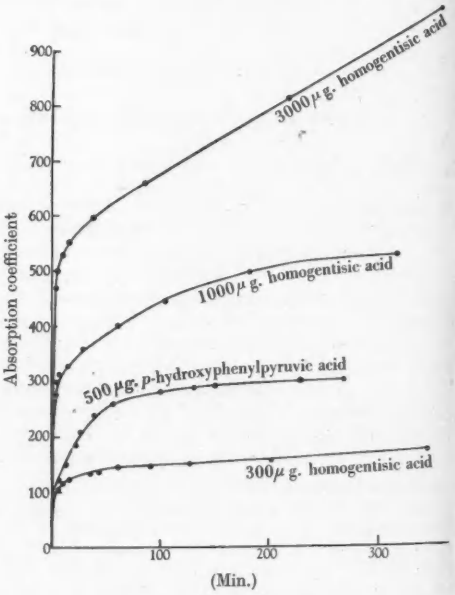

Fig. 1. The rate of colour development in the Briggs reaction with different concentrations of homogentisic acid and *p*-hydroxyphenylpyruvic acid.

the higher concentration the colour increases considerably over several hours and the light absorption observed after 4 hr. is not proportional to the concentration of homogentisic acid. The rate of the development of colour with *p*-hydroxyphenylpyruvic acid was also measured (Fig. 1) and the results obtained were similar to those reported by Medes (1932). The colour development with the keto acid is slower than with homogentisic acid, but the difference is not very great. It appears therefore that a differentiation of the two acids based on separate readings at suitably chosen intervals would be very inaccurate.

Application of Beer's law. At higher concentrations Beer's law is not obeyed, at least if a reading is taken within 5 hr. after mixing the reagents (Fig. 1). However, with concentrations of homogentisic acid varying between 100 and 600 μg./25 ml. the colour produced is linearly proportionate to the concentration. It is advisable therefore to dilute the sample sufficiently so that the final concentration of homogentisic acid should not exceed the value of 600 μg./25 ml.

Recovery of homogentisic acid added to normal urine. Some normal urines give a very slight colour with the Briggs reagent, but the error introduced by the reduction of the molybdate reagent through substances other than homogentisic acid in the urine of an alcaptonuric can be safely neglected. Added homogentisic acid is satisfactorily recovered, though there is a slight deflexion, especially at higher concentrations.

Table 1. *The reaction of various substances with phosphomolybdic acid in acid solution*

(Strongly positive indicates that the intensity of the colour is of the same order as that found with homogentisic acid and that the colour develops within the first 10 min. Slightly positive means that the colour is less than 5% of that of homogentisic acid on a molecular basis and that the appearance is delayed.)

Strongly positive	Slightly positive	Negative
Hydroquinone	Catechol	2:5-Dihydroxyphenylpyruvic acid lactone
Gentisic acid	3:4-Dihydroxyphenylalanine	Cysteine
2:5-Dihydroxyphenylalanine	Uric acid	Glutathione
Ascorbic acid	—	Phenylpyruvic acid
p-Hydroxyphenylpyruvic acid	—	—

Specificity of the reaction. All substances which have two hydroxyl groups in the benzene ring in *p*-position to each other resemble homogentisic acid in its reduction of the molybdate reagent (Table 1). Thus hydroquinone, gentisic acid and 2:5-dihydroxyphenylalanine would be estimated as homogentisic acid. Ascorbic acid, which is also a normal constituent of urine, gives a strongly positive reaction and might account for the slight colour produced with many normal urines. The behaviour of *p*-hydroxyphenylpyruvic acid which has already been discussed above, contrasts with that of phenylpyruvic acid showing that the keto acid group as such does not reduce Briggs's reagent. The lactone of 2:5-dihydroxyphenylpyruvic acid does not reduce, indicating that the lactone ring does not open under the conditions of the test.

If the hydroxyl groups in the benzene are in *o*-position to each other, only a very slow reaction with the Briggs reagent is observed. Thus catechol and dopa will not interfere with this estimation.

PART 2. THE IODOMETRIC METHOD

Experimental

Reagents. The reagents used were those described by Lieb & Lanyar (1929) and Metz (1930).

Procedure. A. *Macromethod.* To 10 vol. of freshly collected urine 1 vol. of approximately 3 N-HCl was added and the sample was stored at 0°. To 10 ml. of acidified urine were added 40 ml. of water, 5 ml. of the 5% KI solution, 5 ml. of the starch solution (1% in 20% NaCl) and 0·55 g. of $NaHCO_3$. If the urine was strongly pigmented, the amount of water was increased. The addition of bicarbonate to the acidified urine produces a buffer system and the pH obtained varied between 7·5 and 7·8. At this slight alkalinity both the destruction of the quinone-acetic acid and substitution by iodine appear to be minimal and the oxidation is sufficiently fast for the purpose.

The solution is then titrated with 0·05 N-iodine solution until a definitely blue colour which persists for 45 sec. is obtained. The rate of titration is adjusted in such a way that a large excess of iodine is avoided. The solution is then acidified with 25 ml. of 7 N-H_2SO_4 and left to stand for 3 min. before titration of the liberated iodine with thiosulphate. If the volume of the solution before the acidification exceeds 70 ml. the amount of acid added has to be increased accordingly. The amount of 0·05 N-thiosulphate used up in ml. multiplied by 4·2 gives the amount of homogentisic acid in the acidified sample in mg.

B. *Micromethod.* The micromethod was used for rat urine and for deproteinized plasma filtrates. The rat urines were acidified in the same proportions as described above for human urines. For the estimation of homogentisic acid in blood, 2·5 ml. of 10% trichloroacetic acid were added to 5 ml. of heparinized plasma and the mixture was centrifuged. The clear supernatant was stored at 0° in a dark bottle and a measured sample used for titrations.

The samples were diluted when necessary, to a volume of 10–12 ml.; 1 ml. each of the KI and starch solution were added and an amount of $NaHCO_3$ to bring the pH to about 7·6. The titration with iodine was done as described above. The solution was then acidified with 5 ml. of 7 N-H_2SO_4 and the liberated iodine was titrated, after standing for 3 min., with 0·01 N-thiosulphate. The colourless solution was then

back-titrated with iodine until a faint blue colour was obtained. The amount of thiosulphate used up minus the amount of iodine consumed in the back-titration in ml. multiplied by 0·84 gives the amount of homogentisic acid in the sample in mg. Microburettes were used for these titrations.

RESULTS

Recovery. The values obtained for solutions of homogentisic acid in water were 98·5–99·5 of the theoretical and the iodine uptake in alkaline solution was found to be equivalent to the amount of iodine liberated on acidification. If homogentisic acid was added to urine the iodine uptake was greater than the theoretical due to the oxidation of other substances, but the amount of iodine liberated on addition of acid was again about 99 % of the theoretical. In the many samples of normal human or rat urine tested without addition of homogentisic acid there was either only a slight liberation of iodine in acid solution or in most cases none at all.

Recovery from plasma, however, was not quantitative. If amounts of homogentisic acid corresponding to concentrations of 5–25 mg./100 ml. were added to plasma, recovery varied between 85 and 95 %, the higher values being obtained with higher concentrations. With metaphosphoric acid as deproteinizing agent, recoveries were slightly worse. It is believed that this loss is due to absorption by the protein precipitate. Moreover most samples of normal human plasma showed a blank corresponding to an apparent homogentisic acid content of 0·5–1·0 mg./100 ml.

Possible interference by compounds containing amino or thiol groups. It appeared possible that the quinoneacetic acid would react with substances containing amino or thiol groups to form *N*- or *S*-substituted hydroquinones (Snell & Weissberger, 1939) which might be further oxidized and certainly not be reduced on acidification. Homogentisic acid was therefore titrated in the presence of varying quantities of urea, glycine and cysteine. Table 2 shows that, though the iodine uptake in the case of cysteine is considerably increased, the consumption of thiosulphate in acid solution is not affected. It is concluded that the compounds do not interfere with the estimation.

Specificity of the reaction (Table 3). Other compounds having hydroxyl groups in 1 : 4-positions in the benzene ring, such as gentisic acid and hydroquinone behave exactly like homogentisic acid and cannot be differentiated from each other by this method. But 2:5-dihydroxyphenylalanine behaves in an atypical fashion. Its iodine uptake in alkaline solution exceeds considerably the expected value of two equivalents and there is only a slight, but variable, liberation of iodine in acid solution. It is likely that when an amino and a quinolic structure exist in the same molecule, internal condensation of the type discussed above, occurs leading to further oxidation and/or substitution. The lactone of 2:5-dihydroxyphenylpyruvic acid is apparently oxidized in alkaline solution by iodine, but again this reaction cannot be reversed by acidification; the amount of thiosulphate used up is only a fraction of the theoretical value.

Table 2. *The titration of homogentisic acid with iodine in the presence of glycine, cysteine and urea*

(In all three experiments 200 μg. of homogentisic acid were added to solutions containing the stated amount of amino-acid or urea.)

Nature and amount of substance added	Theoretical iodine uptake and liberation at acid or neutral pH	Iodine uptake at pH 7·4	Iodine liberation at pH 0
		(ml. of 0·01 N-I_2)	
500 μg. glycine	0·238	0·274	0·240
500 μg. cysteine hydrochloride	0·238	0·602	0·236
500 μg. urea	0·238	0·263	0·240

Table 3. *The titration of several derivatives of hydroquinone and catechol with iodine*

(All titrations were carried out by the micromethod using 0·01 N-iodine and thiosulphate solutions.)

Substance	Amount taken (μg.)	Theoretical iodine uptake and liberation	Iodine uptake at pH 7·4	Iodine liberation at pH 0
			(ml.)	
Homogentisic acid	300	0·357	0·358	0·360
Gentisic acid	300	0·390	0·392	0·399
Hydroquinone	300	0·545	0·540	0·543
Catechol	300	0·545	0·980	0·265
2:5-Dihydroxyphenylpyruvic acid lactone	200	0·224	0·440	0·065
2:5-Dihydroxyphenylalanine hydrate	300	0·279	0·870	0·115
3:4-Dihydroxyphenylalanine	300	0·300	0·770	0·210

It was pointed out above that the reaction of compounds possessing a hydroquinone structure, with iodine in acid and neutral solution respectively, is correlated with the magnitudes of the oxidation-reduction potentials involved and their change with pH. The E_h values of *o*-quinones in the benzene series are higher by about 0·1 V. than those of *p*-quinones. Catechol and its derivatives should therefore, like hydroquinone, consume 2 atomic equivalents of iodine at pH 7·4 and liberate the same amount at pH 0. Catechol at pH 7·0–8·0 has an iodine uptake greater than the theoretical and there is only a slight liberation of iodine on acidification (Table 3). It is known that *o*-quinones are much less stable than *p*-quinones and this in addition to a substitution probably accounts for the results obtained. 3:4-Dihydroxyphenylalanine shows a similar behaviour.

Keto acids, like phenylpyruvic and *p*-hydroxyphenylpyruvic acids consume iodine at slightly alkaline reaction, but do not liberate it on acidification. They do not therefore interfere with the estimation.

PART 3. THE SILVER REDUCTION METHOD

Experimental

Reagents. Gold sol. The following modification of the method of Faraday (1857) was used: to 250 ml. of water were added 6 ml. of a 0·5% solution of gold chloride and 5 ml. of 0·25 N-K_2CO_3 solution. This was followed by the addition of 1 ml. of an ethereal solution of yellow phosphorus prepared by diluting a saturated solution five times. The solution was then allowed to stand for 30 min. and boiled under reflux for 15 min. The gold sol thus produced was red and contained about 70 μg. of gold/ml. It was stored in the dark at 0°. For the estimation of homogentisic acid this solution was diluted ten times.

0·2 N-*Acetic acid.*

0·2 N-*Sodium acetate.*

Gum arabic solution 5%. This was prepared by dissolving gum arabic in hot water and filtering the solution through filtercel.

Silver nitrate solution (A.R.) 1%.

Procedure. To a carefully cleaned test tube or measuring cylinder was added 1 ml. of the acetic acid, 0·5 ml. of the sodium acetate solution, 2 ml. of 5% gum arabic, 1 ml. of 1% $AgNO_3$ and 0·5 ml. of the dilute gold sol. The tube was shaken to ensure complete mixture and 1 ml. of the solution to be tested, which should not contain more than 200 μg. of homogentisic acid, was then added. The solution was then made up to 10 ml. with water and well mixed.

The solutions containing homogentisic acid develop a gold-brown colour and this is read, usually after 2 hr. on a Hilger absorptiometer with a neutral filter. A solution containing all the reagents, but no homogentisic acid, gives a very slight absorption. This is allowed for either by deducting this absorption from the experimental values or by placing the control solution in the compensating cup of the absorptiometer. Concentrations of between 20 and 200 μg./10 ml. of final solution can be estimated under these conditions.

RESULTS

Application of Beer's law. Over the range of 20–200 μg. there is an approximately linear correlation between concentration and absorption coefficient, if the reading is made after 2 hr. (Fig. 2). With much higher concentrations of homogentisic acid the colour of the silver sol is darker, the Tyndall effect becomes more marked and deviations from Beer's law are observed. The two curves in Fig. 2 were obtained with different sets of reagents and though the curves are very similar, they are not identical. It is advisable therefore to prepare a fresh set of standards of homogentisic acid for each series of tests.

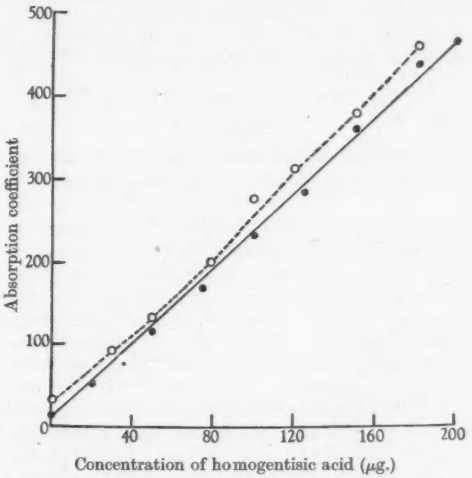

Fig. 2. The relationship between absorption coefficient and concentration of homogentisic acid in the silver reduction method. The two curves were obtained with reagents prepared in a similar manner.

Time factor. James (1939) has shown that the reaction between hydroquinone and silver ions in the presence of gum arabic is an autocatalytic reaction. The same appears to apply to the reaction between homogentisic acid and silver ions. Fig. 3 shows the rate of colour development with two different concentrations of homogentisic acid. With the higher concentration there is little further change after 20 min., but with smaller amounts of homogentisic acid the development of the colour is much slower. It was found that with concentrations above 20 μg./10 ml. the rate of change after 2 hr. is so slow that readings can be safely taken after that time. There is however, a further, very gradual, change;

thus the colour observed after 24 hr. is darker and the absorption coefficient as measured with a neutral filter is further increased. It is likely that this effect is due to a coagulation of the primary particles by the ions present in the solution.

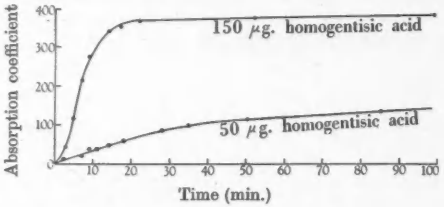

Fig. 3. The rate of colour development with two different concentrations in the silver reduction method.

Application to urine analysis. The presence of large amounts of chlorides makes it impossible to apply the method directly to urine and it was therefore necessary to extract the homogentisic acid into an organic solvent. Homogentisic acid is about six times more soluble in water than in ether and the relative proportions of ether and water had to be chosen accordingly.

Estimation of homogentisic acid in urine. Acidified urine (2·5 ml.) was extracted four times with 20 ml. portions of ether. The ether was freed from peroxides, saturated with water and kept in a dark bottle. For each extraction shaking by hand was carried out for exactly 2 min. at a reasonably constant rate. The combined ethereal solutions amounting to about 120 ml. were dried over anhydrous Na_2SO_4 and filtered into a distilling flask; the Na_2SO_4 was washed carefully and the solution was concentrated to about 15 ml. This solution was then extracted twice with 15 ml. portions and once with a 10 ml. portion of water. Most of the ether was then removed by placing the combined aqueous solutions in a water-bath at 60° for 3–5 min. The volume was then made up to 50 ml. and this solution was used for the estimation of homogentisic acid as described above.

Accuracy of the method. The reproducibility of the colorimetric readings for solutions of homogentisic acid in water was not as good as might be desired. Results obtained with reagent prepared on different days varied within ±5%. Many attempts were made to abolish these discrepancies by further purification of the reagents, careful cleaning of the glassware used, elimination of daylight, etc.; however, we were not able to achieve a degree of reproducibility greater than the one stated above.

With urine, recovery was found to be incomplete and averaged 90%, with a standard variation of about 3%. This incomplete recovery is to some extent due to theoretical limitations; thus with relative proportions of aqueous and ethereal phase used, about 1–1·5% of the homogentisic acid originally present will remain in the aqueous solution. Another source of error is due to unavoidable mechanical losses involved in the extraction, transference of solutions, evaporation, etc. Recovery of added homogentisic acid from plasma varied in different experiments between 78 and 85%. The lower rate of recovery in the case of plasma as compared to urine is probably mostly due to absorption which takes place during the precipitation of the protein.

Specificity of the method. A number of compounds which might be present in urine or plasma reduce silver nitrate under the conditions used in this method, but with most substances tested the reduction is much slower than that observed with homogentisic acid. Thus adrenaline, 2:5-dihydroxyphenylalanine, 3:4-dihydroxyphenylalanine and ascorbic acid produce a yellow colour after some hours. Since these substances, however, are not extracted by ether, they cannot interfere with the method.

The second factor which renders this method so specific is the fact that the rate of the reaction with silver at the pH chosen is rather slow and varies considerably for different compounds. Fig. 4 shows that

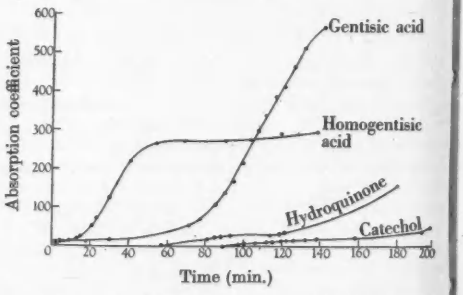

Fig. 4. The rate of colour development with gentisic acid, homogentisic acid, hydroquinone and catechol in the silver reduction method.

the reaction with homogentisic acid is faster than with other hydroquinone derivatives tested. Thus with hydroquinone itself there is hardly any colour developed after 2 hr. and even gentisic acid gives a time curve which is very different from that found with the next higher homologue. The reduction with catechol is very slow indeed. By reading the colour of the test solution after 1 and 2 hr. and comparing the ratio with that given by an authentic specimen of homogentisic acid, an identification of the unknown material is possible. Phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and the lactone of 2:5-dihydroxyphenylpyruvic acid do not react at all with the silver reagent.

DISCUSSION

All the methods discussed in this paper depend essentially on the reducing properties of homogentisic acid. The reduction of phosphomolybdic acid at a very acid pH, which is the basis of Briggs's method, can be brought about in varying degree by a number of other substances which may occur in urine, and the method can therefore only be applied if such compounds are not present in significant amounts relative to that of homogentisic acid. Thus in congenital human alcaptonuria, other aromatic acids metabolically related to tyrosine and phenylalanine are absent (Neuberger *et al.* 1947) and Briggs's method may be expected to give accurate results if the limits of concentration discussed above are observed. However, if the patient receives large doses of salicylates which may give rise to gentisic acid or if the darkening of urine is prevented by medication of massive doses of ascorbic acid, misleading results may be obtained by the phosphomolybdate method. In experimental alcaptonuria in animals and in premature infants the urine may contain appreciable amounts of *p*-hydroxyphenylpyruvic acid; in these cases the Briggs method will yield inaccurate results and it is advisable to use one of the other methods.

The iodometric technique is both convenient and accurate and its specificity is much greater than that of the phosphomolybdate method. It is clear however that any substance having an oxidation-potential smaller than that of the iodine system at 7·0 and greater than that of the iodide system at pH 0 will be titrated as homogentisic acid, provided its reaction with iodine is reversible. In practice, however, this reaction is characteristic of derivatives of hydroquinone and accurate results for the total amount of *p*-quinolic substances will be obtained, unless large concentrations of catechol or catechol derivatives are also present in the solution. The method can also be applied on a micro scale and appears to be the most generally useful analytical method for homogentisic acid.

The reduction of silver ions by homogentisic acid at pH 4·4 after ether extraction is more specific than the other two methods but it is laborious and not very accurate. Its specificity is largely due to the characteristic time curve which is shown by this reaction and which can be observed colorimetrically. It is also clear that any substance having a distribution coefficient between water and ether very different from that of homogentisic acid will be largely eliminated. The fact that even gentisic acid has a time-curve quite different from that of homogentisic acid makes it appear improbable that any other substance which might occur biologically should be mistaken for homogentisic acid, if the colour is plotted against time. The method will therefore be mainly applied, if it is desired to distinguish between homogentisic acid and some other closely related substance. The silver reduction method can also be used for the estimation of small amounts of material.

SUMMARY

1. It is shown that the colour which arises in the reaction of homogentisic acid with phosphomolybdic acid takes some time to develop to its maximum and that the rate of colour development depends on the concentration of homogentisic acid. The colour develops more slowly with *p*-hydroxyphenylpyruvic acid but it is pointed out that the difference is not sufficient for a differential estimation. It is also shown that Beer's law is only obeyed with lower concentrations of homogentisic acid. The reaction is not very specific and has to be used with caution.

2. Experimental conditions for the iodometric estimation of homogentisic acid have been defined which give a recovery of about 99 % in water and urine. Neither amino-acids such as glycine and cysteine nor keto acids like *p*-hydroxyphenylpyruvic acid interfere with the reaction. Hydroquinone and gentisic acid behave towards iodine like homogentisic acid, whilst catechol and dopa behave irregularly and might produce misleading results if present in significant amounts.

3. A new method for the estimation of homogentisic acid has been worked out. The homogentisic acid is estimated by the reduction of silver ions at pH 4·4; the silver is precipitated in the form of a sol of small and fairly uniform particle size in the presence of a gold sol and gum arabic. The time curve of the colour development is highly characteristic of the reducing compound used and permits a very specific estimation of homogentisic acid in the presence of other reducing substances.

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Studies on Alcaptonuria

2. INVESTIGATIONS ON A CASE OF HUMAN ALCAPTONURIA

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The great interest which biochemists have taken in alcaptonuria is largely due to the fact that this metabolic error may be expected to yield information on the normal metabolic pathway of aromatic amino-acids. Such an expectation is based on the assumption that the formation of homogentisic acid is a step in the normal oxidation of phenylalanine and tyrosine and that the defect of the alcaptonuric consists of an inability to break down further this intermediary metabolite. The evidence for this interpretation has been fully presented by Garrod (1923) and has been further strengthened by recent work on the experimental production of alcaptonuria in animals by dietary means. Thus in normal white rats the administration of very large doses of phenylalanine leads to excretion of small amounts of homogentisic acid (Papageorge & Lewis, 1938; Butts, Dunn & Hallman, 1938), whilst a diet deficient in methionine and cysteine (Glynn, Himsworth & Neuberger, 1945) or diets practically free of protein (Neuberger & Webster, 1947) produce a marked alcaptonuria even with a normal intake of aromatic amino-acids. It has also been shown that guinea-pigs excrete homogentisic and *p*-hydroxyphenylpyruvic acids if they are given a diet deficient in ascorbic acid but enriched by tyrosine (Sealock & Silberstein, 1940; Sealock, Perkinson & Basinski, 1941). It is reasonable to assume that these dietary deficiencies produce alcaptonuria by affecting the cellular concentration of an enzyme or other factor necessary for the normal metabolism of tyrosine and phenylalanine rather than by endowing the organism with a new type of metabolic reaction not normally found. It may therefore be inferred that the oxidation involving homogentisic acid represents at least one pathway of the intermediate metabolism of aromatic amino-acids, probably common to all mammals.

The question as to whether all the tyrosine and phenylalanine which is catabolized passes through the homogentisic acid stage has been discussed at great length, and the earlier literature on this point is fully reviewed by Garrod (1923) and by Neubauer (1928). More recent papers indicate that at least in some cases reduction of nitrogen intake produces a disproportionate decrease in homogentisic acid output (Katsch, 1918, 1920; Braid & Hickmans, 1929; Reinwein, 1931; Sachs, 1931; Lorenz, 1937). On the other hand Lieb & Lanyar (1930) found in one case that the ratio of homogentisic acid to nitrogen in the urine remained fairly constant, although protein intake was greatly reduced. A critical examination of existing data on the output of homogentisic acid in relation to the dietary intake of precursors is rendered difficult by several factors. Most workers did not keep either the composition or the total amount of their diet constant during the experiment; they were also handicapped by the fact that the tyrosine and phenylalanine content of the proteins given was not even approximately known at the time. A further difficulty arises from the lack of statistical analysis of the basic data. The output of homogentisic acid by the alcaptonuric is subject to considerable daily variations, independently of the changes in the diet. In an attempt to obtain a greater constancy Langstein & Meyer (1903) proposed the use of the ratio of homogentisic acid to urinary nitrogen (H : N) as a measure of the rate of conversion of tyrosine and phenylalanine to homogentisic acid. The H : N ratio is certainly very useful, if the total protein intake varies from day to

day; but the question, as to whether the variation which still occurs on a constant nitrogen intake is closely related to changes of nitrogen excretion, has to our knowledge never been investigated. The need for a proper statistical analysis is also obvious in the interpretation of the many experiments in which potential intermediates were added to the diet of the alcaptonuric and their rate of conversion to homogentisic acid was deduced from the increase of the *H* : N ratio. The significance of such a change will obviously depend on the variability of this ratio on a constant diet without a supplement.

PLAN OF PRESENT INVESTIGATION

Having access to a case of alcaptonuria, we proposed to ascertain first the degree of daily variation in homogentisic acid output on a diet constant in composition and amount and to investigate to what an extent the variations in the daily output of homogentisic acid and urinary nitrogen are correlated. Knowing the standard variation it was intended to assess more quantitatively than has been possible in the past, to what an extent added tyrosine, phenylalanine or other possible intermediates are converted into homogentisic acid.

In spite of the large amount of work which has been done on the 150–200 cases of alcaptonuria which are described in the literature, there are still some gaps in our knowledge of this condition. Thus, in the experimental alcaptonuria in guinea pigs (Sealock *et al.* 1941) and also in the disturbance of the metabolism of aromatic amino-acids which occurs in premature infants (Levine, Marples & Gordon, 1941) a number of intermediate products of tyrosine metabolism such as keto and hydroxy acids are found in the urine. No systematic search for such substances has to our knowledge been made in cases of hereditary human alcaptonuria.

Another point about which there is a certain amount of confusion is the effect of ascorbic acid administration on human alcaptonuria (viz. Diaz, Mendoza & Rodriguez, 1939; Monsonyi, 1939; Sealock, Galdston & Steele, 1940; Leslie, 1943). We therefore decided to reinvestigate the effect of ascorbic acid on the output of homogentisic acid in our patient. It also appeared of interest to find out whether the alcaptonuric can deal in a normal manner with the extra ascorbic acid administered. This problem presents certain analytical difficulties, since phenol indo-2:6-dichlorophenol which is generally used for the estimation of ascorbic acid has an oxidation potential very close to that of homogentisic acid. We have therefore explored other methods for the estimation of ascorbic acid, applicable to alcaptonuric urines.

In rats alcaptonuria can be produced by giving a diet deficient in methionine and cysteine (Glynn *et al.* 1945) and it appeared possible, at least at the time when our investigations on human alcaptonuria were carried out, that this inborn error might be associated with a disturbance of sulphur metabolism. Such a suggestion appeared to be supported by the report of a single case of combined cystinuria and alcaptonuria (van Amstel, 1910). We proposed therefore to study the effect of the administration of extra cysteine and methionine to the alcaptonuric patient. It may be added, however, that later investigations (Neuberger & Webster, 1947) have thrown considerable doubt on the supposition that the alcaptonuria observed by Glynn *et al.* (1945) was specifically caused by a deficiency of sulphur-containing amino-acids.

The researches of Wolkow & Baumann (1891), Falta (1904), Langstein & Meyer (1903) and especially those of Neubauer (1909) have established the fact that tyrosine and phenylalanine are the precursors of homogentisic acid and have also thrown much light on the intermediate steps involved in these transformations. Thus the observations that *p*-hydroxyphenylpyruvic acid gives rise to the excretion of extra homogentisic acid (Neubauer, 1909), whilst both *o*- and *p*-hydroxyphenylacetic acids are inactive in this respect (Blum, 1908) lead to the conclusion that the introduction of the second hydroxyl group into the phenolic ring and the concomitant shift of the side chain precede the oxidation of the alanine or pyruvic acid to the acetic acid structure. But as Neubauer (1928) points out, it is still uncertain, whether the oxidation proceeds from *p*-hydroxyphenylpyruvic acid through 2:5-dihydroxyphenylpyruvic acid to homogentisic acid or whether 2:5-dihydroxyphenylalanine is the intermediate. Since this amino-acid, which has so far not been tested as a potential precursor of homogentisic acid, was available to us through a convenient synthesis (Neuberger, 1947*b*), we decided to investigate its metabolism in some detail.

In the normal dog homogentisic acid is oxidized to acetone and probably other ketone bodies in the liver, as shown by Embden, Salomon & Schmidt (1906) in perfusion experiments. It is likely that in the normal man also, the liver is responsible for the further breakdown of homogentisic acid. We are, however, quite ignorant as to where this substance is formed or about the mechanism of its excretion by the kidney of the alcaptonuric. The concentration of homogentisic acid in the blood of alcaptonurics has been estimated in the past by a rather unspecific method which was based on the reduction of phosphomolybdic acid in alkaline solution (Katsch & Metz, 1927; Lanyar & Lieb, 1931). The values obtained by this method were of the order of about 3 mg./100 ml. of plasma. On the other hand Abderhalden & Falta (1903) claimed actually to have isolated this acid in the form of its lead salt from 300 ml. of blood taken from an alcaptonuric; the significance of this finding must, however, remain doubtful, since neither the yield, melting point, nor analysis of this lead salt are stated. It appeared desirable therefore to find out by more specific methods (Neuberger, 1947*a*) what the concentration of homogentisic acid in blood of the fasting alcaptonuric is, to compare the concentration in plasma and urine, and thus arrive at some definite conclusions as to transport and renal clearance of this intermediary metabolite.

Lastly we may consider the genetic basis of alcaptonuria. This aspect has been reviewed by Hogben, Worrall & Zieve (1932) who have carried out a careful analysis of all the available data. These authors point out that the findings in the majority of cases are consistent with the hypothesis that this condition is determined by a single autosomal recessive gene substitution. There are, however, a few cases on record in which a dominant mode of transmission is suggested by the fact that alcaptonuria was present both in the parent and in the offspring. It appeared desirable therefore to test the urines of other members of the family for homogentisic acid. If our case belonged to the usual type of alcaptonuria, then both parents and probably other blood relations of our patient must be heterozygous with regard to the gene in question. Now it has been shown that

many genes in man classed as recessive affect the phenotype of the heterozygous individual to some extent. The presence of minute amounts of homogentisic acid in the heterozygous parents might escape detection by the usual qualitative methods, but it was hoped that more sensitive techniques might reveal traces of the acid.

General description of the case

The patient, a girl of 6 years 11 months, was brought to the Diabetic Clinic, University College Hospital, where the presence of unusual reactions with Benedict's reagent and ferric chloride were confirmed.

The mother had noticed that the diapers were stained in early infancy, but she had never been perturbed over the matter and did not seek medical advice, until the patient was 3 years old, when she consulted a physician, because the child was overweight. No observation on the urine was apparently made at that time. The family history will be fully discussed in a later section of this paper. The patient is the only child of elderly parents, the father being 56 years and the mother approximately 45. The weight is in gross excess of normal. Otherwise the personal history presents no unusual features.

On clinical examination the patient looked rather older than her 7 years. Her weight was 82 lb. 5 oz. whereas the expected weight for her age is 47 lb. The height was 50 in. compared with the expected height of 45 in. There was no abnormal pigmentation of the sclerotics and the cartilages of the ears and nose were of normal colour. Further systematic examination revealed nothing abnormal, save in the urine. The patient remained in hospital for about 6 weeks; during that time her weight remained quite constant. She was again readmitted at a later date for a period of 3 days. All the investigations recorded in this paper were carried out during these two periods.

METHODS

Estimation of homogentisic acid. The urine was collected over a 24 hr. period and stored in bottles containing 100 ml. of approximately N-HCl. Estimations for homogentisic acid were carried out in duplicate with the iodometric method; on most samples parallel determinations were also done by the reduction and Briggs's methods (Neuberger, 1947*a*). Agreement between the different methods was always within 5%. For the calculation of homogentisic acid output the iodometric values only were used.

For the estimation of homogentisic acid in plasma, venous heparinized blood samples were centrifuged and deproteinized as quickly as possible. To 5 ml. of plasma were added either 2·5 ml. of a 5% (w/v) HPO_3 solution (1st experiment) or 2·5 ml. of a 20% (w/v) trichloroacetic acid solution (2nd experiment). In the clear filtrates homogentisic acid was estimated both by the micro-iodometric and by the silver-reduction methods (Neuberger, 1947*a*). Results were also checked by the rather unspecific reduction of the Folin phenol reagent (Folin & Ciocalteu, 1927) in alkaline solution using synthetic homogentisic acid as standard.

Determination of ascorbic acid in alcaptonuric urine. It was found, as expected, that homogentisic acid reduced phenol indo-2:6-dichlorophenol in a manner closely similar to that shown by ascorbic acid. However, the end point was not at all sharp, making an accurate determination impossible.

An attempt was made to determine ascorbic acid polarographically, utilizing the anodic wave in a manner similar to that employed in the analysis of fruit juices; interference by homogentisic acid was again encountered. The waves corresponding to the two substances overlapped almost completely under all conditions investigated. Pure homogentisic acid in aqueous solution gives a cathodic wave, but this is suppressed in urine.

The method finally selected for the determination of ascorbic acid in the alcaptonuric urine was based on the dinitrophenylhydrazine procedure of Roe & Kuether (1943); in this method, which is fairly specific (Penney & Zilva, 1945), ascorbic acid is converted into dehydroascorbic acid by shaking with charcoal in the presence of trichloroacetic acid. The method is not sufficiently sensitive for the accurate determination of the ascorbic acid normally present, but it is quite suitable for the estimation of excretion after a test dose.

Diet. The patient's diet was kept constant and all food returned to the kitchen was weighed so that a computation could be made of the daily intake of calories, protein, carbohydrate and fat. Detailed data of the composition of the diet are given in Table 1 which also includes an estimate of the daily intake of tyrosine and phenylalanine, the

Table 1. *Standard diet given to the patient*

(Total number of calories = 1850.)

Food	oz.	Carbohydrate (g.)	Protein (N × 6·25) (g.)	Fat (g.)	Estimated sum of tyrosine and phenylalanine (g.)
Milk	20	30	20	20	2·2
Breakfast cereal	1	24	2	—	0·18
Bread	5	75	10	—	0·95
Biscuit	0·5	8·4	1	3·4	0·09
Egg	—	—	3	0·3	0·30
Butter	1·25	—	—	30	—
Cake	1	15	2	5	0·20
Cheese	1	—	7	10	0·81
Cocoa	0·25	2	2	1·5	0·20
Meat	2	—	9·6	10·8	0·67
Potatoes	5	17·5	2	—	0·20
Cabbage	2	4·8	1·8	—	0·18
Total	—	176·7	60·4	81·0	5·98

calculations being based upon analyses given by Block & Bolling (1945) or other recent publications. Where no figures were available a figure of 10% for the combined two aromatic amino-acids in the protein was assumed. It is realized that these estimates can be only rather rough. When all the diet was consumed it was estimated that the subject received in a day 177 g. of carbohydrate, 60·4 g. of protein, and 81·0 g. of fat, making altogether 1848 calories. The tyrosine and phenylalanine intake was calculated to be 5·98 g./day.

RESULTS

Chemical investigations of the urine

Identification of homogentisic acid. The urine gave the typical reactions with alkali, Benedict's reagent and $FeCl_3$ and the homogentisic acid was identified by isolation.

Urine (1 l.) containing 10% (v/v) of N-HCl was extracted in a continuous extractor with peroxide-free ether for 18 hr. The ether solution was dried over Na_2SO_4, filtered and concentrated to about 50 ml. This ethereal solution was then extracted three times with 40 ml. portions of water and the homogentisic acid present in the combined aqueous solutions was precipitated by the addition of 40 g. of lead acetate trihydrate dissolved in about 50 ml. of hot water. The precipitate was filtered off after standing at 0° for 2 hr. The lead salt was twice recrystallized from water and dried *in vacuo* over $CaCl_2$; it had m.p. 214–215° as reported by Wolkow & Baumann (1891). The free acid was prepared by decomposing an aqueous suspension of the lead salt with H_2S and concentration of the solution *in vacuo* under nitrogen. The moist, crystalline residue was dissolved in ether, the ethereal solution dried and concentrated to low volume until crystallization started. Chloroform was then added and the crystalline material filtered off. It had m.p. 144–145°; the m.p. was not depressed when the material was mixed with a specimen of synthetic homogentisic acid.

Search for other aromatic substances. The urine, after four extractions, each time with 12 vols. of ether, gave no reduction with Benedict's reagent, ammoniacal silver nitrate or Briggs's reagent, indicating the almost complete absence of reducing substances soluble in water and insoluble in ether. The ether solution was then extracted with water as described above; this extraction transferred about 99% of the homogentisic acid back into the aqueous phase. The ethereal solution was then extracted three times with a solution containing 1% $NaHCO_3$. The $NaHCO_3$ solution was then tested for reducing properties; similar tests were carried out on the ethereal phase, after evaporating the ether and taking up the residue in water. Results with both solutions were entirely negative. It follows that the reducing properties found originally in the urine were confined, after the fractionation, to the solution containing the bulk of the homogentisic acid. A comparison of estimations by the Briggs, the iodometric and the very specific silver reduction methods indicated that homogentisic acid was the only reducing agent present in this solution. This assumption was also supported by the fact that the amount of recrystallized lead homogentisate recovered corresponded to a yield of 70% calculated on the basis of the titration data.

Homogentisic acid and 2:5-*dl*-dihydroxyphenylalanine give a yellow-orange precipitate with the Millon reagent; this precipitate is, however, so insoluble that these two substances show no colour in the tyrosine estimation according to Folin & Ciocalteu (1927). Tyrosine, *p*-hydroxyphenylpyruvic and -lactic acids give colours of equal intensity relative to the respective molecular weights (Medes, 1932). Many samples of urine of the alcaptonuric patient were tested by this method with completely negative results. It was concluded that the presence of significant amounts of these substances, i.e. tyrosine and the two phenolic acids, can be excluded.

In another experiment urine was extracted with ether as described above and the ethereal solution extracted with 0·1 N-HCl to remove homogentisic acid. The ethereal phase was then extracted with 1% $NaHCO_3$ and the aqueous extract tested for keto acids by the method of Penrose & Quastel (1937). The result was negative. In a model experiment it could be shown that phenylpyruvic acid added to urine could be recovered to the extent of 90–95% by this method.

The output of homogentisic acid under different conditions

Intake and urinary output of nitrogen. The excretion of nitrogen and of homogentisic acid in the urine of the patient were estimated over a period of 32 days. The basal diet, as given in Table 1, was not consumed completely on some days, with the result that the average calculated daily N intake amounted to 9·18 instead of 9·82 g. The values for N intake cannot be considered very accurate, since these were based, not on actual estimations, but were calculated from the tables of McCance & Widdowson (1940). The average daily N excretion in the urine during the experimental period was 9·16 g. with a standard deviation of 1·14. Since the faecal N may be assumed to be about 5–10% of the total N output, it is calculated that the patient excreted on the average 9·6–10·0 g. N/day. It would thus appear that the patient was in a negative N balance during the investigation, losing about 0·4–0·8 g. N/day. We believe, however, that such a conclusion is unwarranted, especially, since the body weight did not change significantly during this period. It is more likely that the method of calculating N intake from food tables is not sufficiently accurate for our purpose, or that the patient consumed small additional quantities of protein without our knowledge. It is probable, therefore, that the calculated values both of total N intake and of potential homogentisic acid are too low by about 5–10%.

Variations in the daily output of homogentisic acid and in the H : N ratio. Since the composition of the

Table 2. *Daily excretion of homogentisic acid and of* N *of the patient on the basal diet only*

Date	Vol. of urine (ml.)	Homogentisic acid output (g.)	Total N (g.)	H : N ratio
26–27 Jan.	640	4·87	9·1	0·535
27–28 Jan.	885	4·39	8·9	0·495
28–29 Jan.	715	4·65	9·75	0·477
30–31 Jan.	1135	5·14	9·94	0·517
31 Jan.–1 Feb.	1175	5·09	9·26	0·55
1–2 Feb.	1015	3·93	8·74	0·45
4–5 Feb.	1040	5·01	9·98	0·50
5–6 Feb.	975	4·19	8·73	0·48
6–7 Feb.	750	3·57	7·02	0·50
10–11 Feb.	780	5·12	9·99	0·512
11–12 Feb.	1290	5·23	11·19	0·468

diet did not vary at all from day to day and the quantity of food eaten remained nearly constant, it was expected that the output of homogentisic acid would vary very little. Table 2 which gives the relevant data for a representative 10-day period, shows that the fluctuations both of the daily output of homogentisic acid and of the H : N ratio were quite marked. The mean value of the output for the 32-day period was 4·66 g./day with a standard deviation of 0·634. The coefficients of variation for the excretion of N and of homogentisic acid were similar, 12·5% in the former and 13·6% in the latter.

These variations in the excretion of homogentisic acid are, however, associated with similar changes in daily N output. Thus the H : N ratio averaged 0·495 with a standard deviation of 0·05; the coefficient of variation was 10·4%, definitely lower than the corresponding values for H and N alone. Moreover, the correlation coefficient R between daily output of H and N was found to average 0·793, which is 4·4 times the standard error. This figure is statistically significant. It follows that even with a constant diet the H : N ratio provides a more reliable basis for the interpretation of metabolic experiments than the absolute values of homogentisic acid output. But it is also clear that this correlation between H and N is not complete and that part of the variability of homogentisic acid excretions is caused by factors not affecting output of N.

The extent of the conversion into homogentisic acid of the tyrosine and phenylalanine present in the basal diet. It was calculated (Table 1) that the basal diet contained about 6 g. of phenylalanine and tyrosine if fully consumed, which could give rise to a maximal daily excretion of 5·8 g. of homogentisic acid. When allowance is made for dietary items rejected on several days, this average figure is reduced to about 5·5 g. This value is only approximate. As was pointed out above, the patient probably consumed about 10% more protein than our calculations indicate; moreover about 5–10% of the aromatic amino-acids ingested are likely to be excreted in the faeces. However, these two sources of error largely cancel each other and, if it is assumed that the ratio of potential homogentisic acid in the diet to total dietary N equals that ratio in the urine, then it follows that about 84% conversion has taken place. This follows from the fact that the calculated average dietary and urinary N are almost equal; the maximal potential homogentisic acid is therefore 5·5 g. whilst the mean value for the daily excretion was 4·66 g. A very similar figure is obtained if the H : N ratios in the diet and in the urine are compared. The theoretical value for maximum conversion is 0·592 whilst the experimental figure is 0·495; from this calculation the rate of conversion is 83·6%. This rather close agreement between the two calculations is rather illusory since both methods assume that the relative composition of the diet with respect to phenylalanine and tyrosine is known accurately. This is not the case; the analytical uncertainty is estimated to be about 5–10%. It is concluded from this investigation that 80–100% of the tyrosine and phenylalanine present in the basal diet and catabolized by the patient are excreted as homogentisic acid in the urine; it is probable, that under the conditions used, the conversion is almost, but not quite, complete.

Effect of feeding additional l(+)*-phenylalanine on the output of homogentisic acid.* In order to gain further information as to the completeness of the conversion, 3 g. of *l*(+)-phenylalanine were administered to the patient in addition to the basal diet, in three separate experiments. The amino-acid was given in a 1% solution at 10 a.m. and the urine was collected over the next 24 hr. It will be shown later that it can be safely assumed that the extra homogentisic acid is excreted in the first 6–8 hr. The results (Table 3) indicate clearly that the ingestion of the extra phenylalanine produced an increase, both in the output of homogentisic acid and in the H : N ratio. The values of the daily homogentisic acid output found in the three experiments are between 7·0 and 7·4 g.; they differ from the mean of the control series by more than three times the standard deviation. The H : N ratios are even more conclusive; they differ from the mean value on the basal diet alone by more than five times the standard deviation. There is therefore no doubt that feeding phenylalanine leads to an increased excretion of homogentisic acid by the alcaptonuric. But these three experiments do not allow us to arrive at a

definite conclusion as to what proportion of the amino-acid is converted into homogentisic acid. The calculated values in Table 3 were obtained by first correcting the total urinary N for the N derived from the extra phenylalanine. That fraction of the homogentisic acid due to the aromatic amino-acids contained in the basal diet was then calculated by multiplying the corrected N figure by 0·495. The difference between the total homogentisic acid and the calculated value of the fraction derived from the basal diet was ascribed to the extra phenylalanine fed. The mean value for this extra homogentisic acid in the three experiments was 2·67 g., the standard error of the mean being 0·088; complete conversion would require 3·03 g. The fiducial limits of error for $p = 0·95$ are 2·29 and 3·055. The results are thus consistent with any hypothesis which requires a conversion of between 75–100 %.

Table 3. *Effect of the addition of l(+)-phenylalanine to the basal diet on the daily excretion of homogentisic acid*

(The patient was given 3 g. of the amino-acid in addition to the basal diet; this could give rise to a maximal additional excretion of 3·03 g. of homogentisic acid.)

	Test no. 1	Test no. 2	Test no. 3
Total homogentisic acid excreted (g./day)	7·17	7·40	7·02
Total daily N (g.)	9·39	9·35	9·15
H : N ratio	0·764	0·791	0·767
Extra homogentisic acid (g.)	2·6	2·85	2·57
% Conversion	85	94	84·8

Effect of the administration of ascorbic acid on the output of homogentisic acid. The output of homogentisic acid and the *H* : N ratio did not change significantly, when the patient received 1 g. of ascorbic acid/day for two consecutive days (Table 4). It also appears that the proportion of ascorbic acid excreted in the urine indicates that the alcaptonuric responds in a normal manner to the large dose of the vitamin administered.

Effect of the administration of cysteine and methionine on the output of homogentisic acid. Neither the ingestion of fairly large doses of methionine nor of cysteine alter significantly the homogentisic acid output or the *H* : N ratio (Table 5). In one experiment this ratio dropped considerably on the day after cysteine had been administered; but this observation could not be repeated and it seems unlikely that this decrease was caused by the administration of the amino-acid.

Renal clearance of homogentisic acid

Two experiments were carried out in which 3 g. of *l*(+)-phenylalanine were administered to the patient by mouth and the concentration of homogentisic

Table 4. *Excretion of homogentisic acid and ascorbic acid on administration of ascorbic acid on two consecutive days*

Date	Time	Dose of ascorbic acid (mg.)	Vol. of urine (ml.)	Homogentisic acid excreted (mg./24 hr.) (10 a.m.–10 p.m.)	*H* : N ratio	Ascorbic acid excreted (mg./24 hr.) (10 a.m.–10 p.m.)
2–3 Feb.	2 p.m.	250	975	4·20	0·477	224·3
	6 p.m.	250				
	10 p.m.	250				
	2 a.m.	250				
3–4 Feb.	2 p.m.	250	1000	4·35	0·579	581·0
	6 p.m.	250				
	10 p.m.	250				
	2 a.m.	250				
4–5 Feb.	—	Nil	1140	5·01	0·50	68·4
5–6 Feb.	—	Nil	1060	4·19	0·48	4·2

Table 5. *The effect of the administration of cysteine and methionine on the excretion of homogentisic acid*

Date	Supplement	Urine vol. (ml.)	Homogentisic acid excreted (g.)	Urinary N (g.)	*H* : N ratio
2–3 Feb.	6·5 g. *l*-Cysteine HCl	940	3·62	7·59	0·477
3–4 Feb.	Do.	1200	4·99	9·23	0·532
4–5 Feb.	Nil	1025	3·10	7·99	0·388
14–15 Feb.	Nil	880	4·32	9·40	0·459
15–16 Feb.	6 g. *l*-Cysteine HCl	1610	5·31	11·01	0·483
16–17 Feb.	Do.	1025	5·56	10·06	0·550
17–18 Feb.	Nil	1200	5·76	10·56	0·545
20–21 Feb.	8 g. *dl*-Methionine	1590	5·01	10·70	0·467
21–22 Feb.	Do.	1300	4·26	9·72	0·438
22–23 Feb.	Do.	1135	5·34	10·40	0·513

acid was measured at suitable intervals in plasma and urine. In the first test about 1·5–2 pints of liquid in the form of lemonade were given in the first few hours after the administration of the amino-acid, whilst in the second test no additional water was supplied.

however, that homogentisic acid added to blood and incubated for a few hours at 37°, can be recovered by the methods used, to the extent of 70–75%, renders this assumption unlikely.

Renal clearance volume. If it is assumed that the reduction observed in these plasma extracts is due to homogentisic acid, renal clearance volumes can be calculated (Table 6). It can be seen that the values obtained are reasonably constant, varying between 400 and 500 ml. A substance which is filtered by glomeruli and not at all reabsorbed may be expected to show a clearance volume of about 70–80 ml. or at the most 100 ml. in our patient. It follows therefore, that if homogentisic acid is not elaborated in the kidney, but transported there by the blood, glomerular filtration alone cannot account for its rate of excretion. We have to assume that the acid, or at least most of it, is actively secreted by the tubuli.

Table 6. *Concentrations of homogentisic acid in plasma and urine after the administration of* 3 *g. of* l(+)*-phenylalanine by mouth*

(In the first test the amino-acid was given at 12 noon and in the second at 10 a.m.)

	Time	Vol. of urine (ml.)	Urinary N in sample (g.)	Homogentisic acid in sample (g.)	*H* : N ratio in urine	Extra homogentisic acid (g.)	Concentration of homogentisic acid (mg./100 ml.) In urine	Concentration of homogentisic acid (mg./100 ml.) In plasma	Renal clearance volume (ml.)
Test 1	12.00	267	0·658	0·305	0·463	—	114	1·7	210
	1.30	242	0·531	0·801	1·51	0·538	331	—	—
	2.00	—	—	—	—	—	—	3·2	320
	3.00	767	0·698	1·26	1·81	0·92	164	4·2	360
	4.00	247	0·418	0·693	1·68	0·487	281	2·8	400
	5.30	277	0·834	0·698	0·837	0·285	252	—	—
	6.00	—	—	—	—	—	—	2·0	375
Test 2	10.00	542	4·965	2·931	0·59	—	541	2·7	—
	11.30	290	0·672	0·987	1·48	0·66	345	2·94	380
	1.30	106	0·838	1·721	2·05	1·32	1624	2·7	520
	4.20	80	1·10	1·115	1·01	0·56	1394	1·26	540

Rate of excretion of homogentisic acid in the urine. Table 6 shows that in both tests the *H* : N ratio rose abruptly 1·5 hr. after phenylalanine was fed, reached a maximum after about 3–4 hr. and then again decreased. The extra homogentisic acid due to the phenylalanine fed was calculated on the assumption that the normal *H* : N ratio was 0·50. In the first test the large amount of water given produced a diuresis, leading to low concentration of homogentisic acid in the urine; the extra homogentisic acid was almost all excreted in 4 hr. In the second test there was no diuresis, the urine was very concentrated and the excretion of homogentisic acid was apparently more delayed. About 80–85% of the dose administered appeared as homogentisic acid in the urine in the 6 hr. period following the administration of phenylalanine.

The concentration of homogentisic acid in plasma. The fasting level of homogentisic acid in the plasma was about 3 mg./100 ml.; the plasma concentration did not rise markedly after administration of phenylalanine. These concentrations are so low as to be near the lower limits of the applicability of the method, moreover, as was pointed out above, minute amounts of reducing substances reacting like homogentisic acid are present even in plasma extracts obtained from non-alcaptonuric patients and the plasma homogentisic acid values reported here can therefore not be considered as very accurate. The possibility that homogentisic acid is bound to plasma proteins and is not liberated by treatment with acid in the cold has been considered. The fact,

The conversion of 2:5-dl-*dihydroxyphenylalanine to homogentisic acid*

Altogether three experiments have been carried out to test the possibility whether 2:5-dihydroxyphenylalanine is an intermediate in the formation of homogentisic acid by the alcaptonuric. In the first test, a total of 3·5 g. of the racemic compound was given in four equal doses at 4 hr. intervals beginning at 10 a.m. The urinary excretion of N for the 24 hr. period was 9·33 g. and that of homogentisic acid 5·6 g.: the *H* : N ratio was therefore 0·606, i.e. higher than the mean of the control period by exactly twice the standard deviation. On the following day the *H* : N ratio had again dropped to 0·53. In the second experiment 4 g. of the amino-acid was given, also in divided doses; the *H* : N ratio for the 24 hr. period was 0·59, whilst on the following day it was still 0·66. These results suggested, but did not conclusively prove, that an increase of homogentisic acid excretion had been produced by feeding 2:5-dihydroxyphenylalanine. The observed increases in the *H* : N ratio

were on the borderline of statistical significance. Examination of the urine suggested that part of the amino-acid was excreted in the urine. Thus the urine after exhaustive ether extraction at pH 1·5 exhibited strong reducing properties and turned dark on addition of alkali. The concentration of the amino-acid was estimated by making use of the fact that 2:5-dihydroxyphenylalanine reduces Folin's phenol reagent about 2·2 times as strongly as tyrosine on a molecular basis. The colour produced with the ether-extracted urine on the days preceding the test was fairly constant and was equivalent to about 0·5 g. of tyrosine. On the day the amino-acid was fed and also on the following day, the value increased to 1·7 and 1·0 g. respectively (in terms of tyrosine), to return again to the value of 0·6 g. on the third day. Similar results were obtained in the second experiment. It is likely that the ether-insoluble reducing substance which is excreted is the *d*-form of the amino-acid and it appears that about 40% of the whole dose given is excreted unchanged.

In view of these findings it was felt that a more decisive result could be obtained by giving the whole of the amino-acid in one dose and estimating homogentisic acid and N content of the urine at short intervals afterwards. The results of this test (Table 7) show that a rise of the H : N ratio to a value of 1·2 was observed on feeding 2:5-*dl*-dihydroxyphenylalanine. It is therefore certain that this amino-acid can be converted to homogentisic acid in the same way as phenylalanine, i.e. by oxidation. With phenylalanine the increased H : N ratio is already found in the first urine sample obtained after 1·5 hr. and the extra excretion of homogentisic acid is completed within 4–6 hr.; with dihydroxyphenylalanine the first urine sample shows a normal H : N ratio and the excretion of homogentisic acid is delayed and apparently not completed after 6·5 hr. Moreover the conversion of *l*-phenylalanine to homogentisic acid is almost complete; with the dihydroxy-compound this is not the case. The latter amino-acid was administered in the racemic form and as hydrate; 3 g. can therefore give rise to 2·34 g. of homogentisic acid if both optical forms are converted, and to 1·17 g. if only the *l*-form can be thus oxidized. The extra homogentisic acid which has been formed in the first 6·5 hr. has been calculated from the data given in Table 7, to amount to 0·8 g. i.e. 35 or 70% respectively of the theoretical value. It is likely that this value would have been slightly greater if the collection of urine had been continued beyond the 6·5 hr. period.

Table 7. *The conversion of* 2:5-*dihydroxyphenylalanine to homogentisic acid*

(The patient received 3 g. of *dl*-2:5-dihydroxyphenylalanine hydrate at 10 a.m. and urine samples were obtained later in the day at the times stated.)

Time	Vol. of urine (ml.)	Total amount of homogentisic acid in sample (g.)	Total N in sample (g.)	H : N ratio	Extra homogentisic acid excreted (g.)
10.00	130	0·515	0·918	0·561	—
11.30	445	0·326	0·554	0·588	0·029
1.00	85	0·462	0·378	1·222	0·289
2.30	50	0·496	0·409	1·213	0·320
4.30	145	0·609	0·661	0·921	0·298

Family history of patient and examination of the urines of relatives

There was no proven consanguinity between the parents, though the maiden name of the mother was the same as the name of the father. The parents, three aunts and an uncle were asked whether they recalled any history in their families of the passage of dark urine, arthritis, bluish staining of the cartilages or stone in the kidney. The replies were all completely negative.

Samples of morning urine of the parents and of 7 other relatives of the patient were examined for homogentisic acid by a slight modification of the silver-reduction method: 10 ml., instead of 2·5 ml., of acidified urine were extracted with altogether 480 ml. of ether, the ethereal solutions were concentrated to 5 ml. and the final aqueous extracts made up to 20 ml.; 5 ml. of this solution, which were equivalent to 2·5 ml. of urine, were used for the estimation. An amount of homogentisic acid of 3–4 μg./ml. of urine would just give a positive reading. The results, with one exception, were all negative and it was concluded that if any homogentisic acid was excreted by these subjects, it amounted to less than 5 mg. a day.

However, the urine of one cousin of the patient's mother gave distinctly positive reactions for homogentisic acid; one sample containing 280 ml. of urine gave a value of 33 mg. by the iodometric method and a value of 31 mg. by the silver-reduction method. The output of this reducing substance increased four to fivefold in the first 4 hr. after the ingestion of 3 g. of *l*(+)-phenylalanine, to return to a lower value again after 5 hr. An attempt was made to isolate this material; 1150 ml. of acidified urine containing 550 mg. of this substance (in terms of homogentisic acid) were extracted with ether in a continuous extractor for 36 hr. and the ether extract was then concentrated to low volume. It was not possible to isolate either homogentisic acid or its very characteristic lead salt. Since homogentisic acid in similar concentrations can easily be isolated from urine with the technique employed, it seemed unlikely that the reducing substance in this urine was homogentisic acid. This conclusion was strongly supported by experiments carried out by Dr C. E. Dent in which partition chromatography was employed and the reaction with silver nitrate and ammonia was used to locate the different bands. Homogentisic acid gave an R_F value of 0·55 (both in urine and in

water), gentisic acid a value of 0·39 and the unknown substance a value of 0·44. Addition of homogentisic acid to this urine produced two distinct bands, whilst with gentisic acid, slight overlapping was observed. Dr Dent concluded that the unknown substance was neither gentisic nor homogentisic acid. The subject had not been taking drugs containing salicylic acid in the weeks immediately before this investigation was carried out, but was in the habit of taking aspirin and had done so for some months previously.

DISCUSSION

Chemical examination of the urine of the patient has revealed the absence of any substances other than homogentisic acid, likely to arise from the metabolism of tyrosine and phenylalanine. Human congenital alcaptonuria differs in this respect from the metabolic disturbance in aromatic amino-acid metabolism occurring in premature infants and also from the experimental alcaptonuria in guinea-pigs, both of which are caused by a dietary deficiency of vitamin C. It is most unlikely that human congential alcaptonuria is caused by, or associated with, an abnormality in the metabolism of ascorbic acid. In our patient at least, administration of large doses of ascorbic acid had no detectable influence on homogentisic acid output, nor was there any evidence of any inability to utilize this vitamin.

Degree of conversion of phenylalanine and tyrosine into homogentisic acid

As to the quantitative conversion of phenylalanine and tyrosine to homogentisic acid, our investigations have not produced an absolutely conclusive result. It has been shown that the excretion of homogentisic acid varies considerably from day to day, even on a diet kept almost constant. Statistical methods have therefore to be used, if the mean daily output and its variability are to be assessed. It would follow, in the light of the results reported here, that many of the observations recorded in the literature on the conversion of aromatic substances to homogentisic acid, are open to serious criticism. The main difficulty which is met in the quantitative interpretation of the data, is the uncertainty concerning the amino-acid composition of the proteins of the diet. However, it is believed that the intake of phenylalanine and tyrosine has not been overestimated, but rather the reverse, and we consider it very probable that, with the mixed diet used in this investigation, only about 80–85 % of the total amount of the two aromatic amino-acids is converted by the alcaptonuric into homogentisic acid. Most of the H : N ratios found by other workers vary between 0·40 and 0·48, with diets similar to ours, and it is likely that a conversion rate of 70–90 % is usual with most alcaptonurics.

The tests in which extra phenylalanine was given, were again not quite decisive; as in order to obtain statistically conclusive results, the number of such tests would have had to be larger, but they also point to a conversion rate of about 80 %. It would be useless to discuss in detail all the many observations carried out on the conversion of extra phenylalanine and tyrosine into homogentisic acid, reported in the literature. It may be said, however, that all workers agree that with *l*-phenylalanine and *l*-tyrosine the conversion is either complete or nearly complete when small doses are given (Falta, 1904; Papageorge, Fröhlich & Lewis, 1938). With bigger doses, such as 15 g., the conversion is incomplete; this was already shown by Mittelbach (1901). It follows therefore that in the alcaptonuric the oxidation to homogentisic acid represents, under conditions of a mixed diet, the main, but most probably not the only catabolic pathway of the two amino-acids, tyrosine and phenylalanine, and the same probably applies to the normal individual.

Such a conclusion that other pathways exist, but are quantitatively of minor importance, is supported by all the other information we possess on the metabolism of aromatic amino-acids. Thus the conversion of phenylalanine to tyrosine which was first suggested by Embden & Baldes (1913) from perfusion experiments and later conclusively demonstrated by Moss & Schoenheimer (1940), using the isotope technique, is probably the main pathway of phenylalanine catabolism in mammals, but an alternative degradation, not leading through tyrosine, is by no means excluded.

As for tyrosine itself, we may assume, that the major part of this amino-acid is further oxidized in the *para* position to the original hydroxyl group. The main evidence for this supposition is obtained from the experiments discussed above, in which alcaptonuria was produced in laboratory animals by dietary means, and also from the work on congenital human alcaptonuria. There must, however, be other pathways. Thus the fact that neither adrenaline nor dopa are dietary essentials, strongly suggests that these substances are formed in the body from tyrosine. In the case of dopa, such a reaction has been clearly demonstrated by Medes (1932) although the claim that this oxidation is reversible cannot as yet be accepted. It follows that, apart from the *para*-oxidation, the body can introduce a second hydroxyl group into the position *ortho* to the phenolic group of tyrosine. It seems, however, that the capacity of the organism to effect this type of oxidation and to deal further with 3:4-dihydroxyphenylalanine is limited. Thus Guggenheim (1913), who ingested 2·5 g. of this amino-acid himself, experienced toxic symptoms, and found that his urine gave positive catechol reactions. Ingestion of tyrosine even on a larger scale would not produce untoward subjective effects, nor would it lead to the excretion of catechol-like substances in the urine. Medes (1932)

also found that, when dopa was fed to her case of tyrosinosis, some of the amino-acid was recovered from the urine, and Fromherz & Hermanns (1914) reported that dopa was incompletely metabolized by the rabbit and not converted into homogentisic acid by the alcaptonuric. All these observations are compatible with the hypothesis that the body forms dopa from tyrosine by *ortho*-oxidation at a slow rate and that it can deal only with small amounts of this oxidation product.

Another pathway of tyrosine metabolism is the introduction of iodine into the phenolic ring which occurs in the formation of di-iodotyrosine and thyroxine. When di-iodotyrosine is fed to rabbits, a large amount is excreted as 3:5-di-iodo-4-hydroxyphenyllactic acid, whilst some inorganic iodide is also found in the urine (Foster & Gutman, 1930). It is most unlikely that any di-iodotyrosine is reduced again to tyrosine. This follows from the observations of Falta (1904) who fed 3:5-dibromotyrosine and iodinated casein to an alcaptonuric and found no change in the output of homogentisic acid. Abderhalden, Bloch & Rona (1907) fed 3:5-di-iodotyrosine to an alcaptonuric without increase of the $H:N$ ratio, but their result is not conclusive since the 2 g. of the iodine compound fed could have caused only a statistically insignificant increase of homogentisic acid production. However, it is clear that the iodination of tyrosine represents a third, quantitatively limited, pathway of metabolism, not leading through the homogentisic acid stage.

Tyrosine may be catabolized by other mechanisms such as decarboxylation to tyramine; but it is very probable that both in man and other mammals *para*-oxidation is the major pathway and this is blocked completely in the alcaptonuric. In this sense the metabolic error in alcaptonuria is complete. The numerous observations which indicate that the $H:N$ ratio drops if the nitrogen intake is reduced or if a ketogenic diet is given, may be explained by assuming that with a low nitrogen intake, alternate mechanisms become quantitatively more important. It is also possible that certain changes in the diet cause a greater diversion of tyrosine into pathways not involving homogentisic acid.

The intermediate steps involved in the oxidation of tyrosine to homogentisic acid

Current ideas on the exact chemical changes taking place in the conversion of tyrosine to homogentisic acid are mainly based on the work of Neubauer (1909) who found that both *p*-hydroxyphenylpyruvic and 2:5-dihydroxyphenylpyruvic acid increase homogentisic acid output in alcaptonuria. In an earlier paper (Neubauer & Falta, 1904) it was shown that both β-phenyllactic and phenylpyruvic acid are converted to homogentisic acid by the alcaptonuric; with the keto acid the conversion was almost quantitative. It was assumed that tyrosine was first transformed to the keto acid which was then further oxidized in the *para* position to 2:5-dihydroxyphenylpyruvic acid; this acid would then give homogentisic acid on decarboxylation. However, the evidence for this sequence of reactions is not conclusive. We know now that the oxidation of amino-acids to keto acids is a biologically reversible reaction and the apparent conversion of *p*-hydroxyphenylpyruvic acid to homogentisic acid might have occurred through tyrosine. Moreover, the increase in homogentisic acid excretion observed on feeding *p*-hydroxyphenylpyruvic acid was much lower than expected and Fromherz & Hermanns (1914) with another alcaptonuric found only a conversion rate of 35%.

Investigations have shown that 2:5-dihydroxyphenylalanine gives rise to increased excretion of homogentisic acid, presumably by being itself converted to that substance; but again this conversion is not quantitative. Both with tyrosine and phenylalanine the conversion appears quantitative only with the *l*-forms (Falta & Langstein, 1903; Lanyar, 1942). It is probable that in our investigations some of the *d*-form was excreted in the urine, as shown by the reducing properties of the fraction from which homogentisic acid had been removed. It is difficult therefore to assess the quantitative significance of our findings. We can only conclude that an appreciable part, probably more than half, of the ingested *l*-form of dihydroxyphenylalanine has been converted to homogentisic acid. Thus it follows that oxidation of tyrosine to 2:5-dihydroxyphenylalanine followed by oxidative de-amination and decarboxylation represents at least one and possibly the most important pathway in the formation of homogentisic acid.

Excretion of homogentisic acid

Estimations of homogentisic acid in the plasma of the alcaptonuric have confirmed earlier observations that the concentration of this substance in the blood is extremely low, even after the giving of extra phenylalanine. In fact, the results are such that we cannot be quite sure that the reduction observed is due to homogentisic acid. There are two possible explanations for the low plasma and high urine concentrations and the consequently high apparent clearance volumes. It is possible that homogentisic acid is made mainly in the kidney and does not pass into the blood in significant amounts. The well-known fact that most alcaptonurics develop in later life ochronosis indicates that traces of homogentisic acid do escape into the general circulation, but the bulk may be directly secreted into the tubuli. The other possibility is that homogentisic acid is made in an organ other than the kidney, appears in the blood, but is actively secreted by the

tubuli at a rate comparable to that shown by substances like di-iodone. Almost all substances so far known to have clearance volumes of a magnitude similar to that of renal blood flow are dyes and not intermediates in normal metabolism. The observation that a substance which arises in the metabolism of a normal dietary constituent should be secreted at a rate approaching that of the renal blood flow would be of general interest.

A decision as to which of the two explanations is correct cannot be made on the basis of the facts so far available. The observations of Katsch & Metz (1927) who administered homogentisic acid intravenously to an alcaptonuric without materially increasing the plasma concentration, would indicate that the material is excreted rapidly by the kidney from the blood. The amount given, however, was small and a repetition of such experiments with both normal people and alcaptonurics is very desirable, especially since it is not impossible that the abnormality of the alcaptonuric may consist of this high rate of renal secretion which removes homogentisic acid from the body before it can be further metabolized. If large differences between normal and alcaptonuric individuals in respect to renal clearance of homogentisic acid were indeed found, this would indicate that alcaptonuria is not due to the absence of an enzyme, but is caused by an abnormality in the secretory function of the kidney.

The genetics of alcaptonuria

The negative results obtained with the urines of both parents prove clearly that this case does not belong to the rare category of alcaptonuria which appears to be dominant. The fact that no traces of homogentisic acid could be found in the urines of the parents and other relatives, some of whom would be heterozygous with respect to the alcaptonuria gene, shows that this condition is truly recessive. The presence of a reducing substance resembling homogentisic acid in one of the urines tested is difficult to explain. This substance which appears to be a derivative of hydroquinone was found in several specimens of urine obtained from the subject and its concentration was greatly increased by ingestion of phenylalanine. It is likely, therefore, that the substance is formed during the metabolism of aromatic amino-acids, though the amount excreted corresponded only to a small fraction of the phenylalanine given. It is doubtful whether there is any connexion between the alcaptonuria in our patient and the excretion of this substance in this one relative, especially since the findings in the other members of the family were so completely negative.

SUMMARY

1. Chemical and other findings are reported in a case of alcaptonuria. It was shown that, apart from homogentisic acid, no other aromatic substances likely to arise in the intermediary metabolism of tyrosine and phenylalanine are excreted in abnormal and detectable quantities.

2. The variability of the daily excretion of homogentistic acid was investigated and it was found that even on a constant diet both the daily output of homogentistic acid and the H : N ratio vary considerably. It is pointed out that the extent of this variability has to be ascertained before any quantitative conclusions can be drawn from experiments in which the conversion of potential intermediates to homogentisic acid is investigated.

3. It was shown that phenylalanine and tyrosine are converted to homogentisic acid by the alcaptonuric to the extent of probably about 80–85%. Catabolism by other pathways is discussed and it is concluded that, though these undoubtedly exist, they cannot account for more than 5–20% of the total aromatic amino-acids metabolized, at least under the conditions of ordinary mixed diet.

4. Neither the ingestion of ascorbic acid, nor of cysteine or methionine had any significant effect on the excretion of homogentisic acid. It was also shown that the alcaptonuric can deal with large doses of ascorbic acid in a normal manner.

5. Ingestion of *dl*-2:5-dihydroxyphenylalanine produced an increased excretion of homogentisic acid; it is suggested that tyrosine is normally first oxidized to 2:5-dihydroxyphenylalanine which is then converted to homogentisic acid through the keto acid.

6. The concentration of homogentisic acid in the plasma of the alcaptonuric was found to be very low, even after the giving of phenylalanine. The high renal clearance volumes found suggest that homogentisic acid is either formed in the kidney or actively excreted by the tubuli at a very high rate. The possibility that alcaptonuria may be due to an abnormality in the secretory function of the kidney is mentioned.

7. No trace of homogentisic acid was detected in the urines of other members of the family. One relative, however, excreted small amounts of a reducing substance which was not homogentisic acid, but which increased on ingestion of phenylalanine.

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Studies on Alcaptonuria

3. EXPERIMENTAL ALCAPTONURIA IN RATS

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It was shown recently (Glynn, Himsworth & Neuberger, 1945) that young albino rats reared on a diet devoid of both cystine and methionine excreted a urine which darkened on standing; incorporation of either of the two sulphur-containing amino-acids in the diet prevented the formation of this pigment or its precursor. The nature of the substance responsible for the darkening of the urine was not definitely established, but positive reactions for homogentisic acid were obtained. It was the primary purpose of the investigation reported in this paper to identify and isolate the substance and to determine the exact conditions which give rise to its excretion.

EXPERIMENTAL

Animals and their general care. In the first experiment the Institute strain of black and white rats were used. Since no alcaptonuria could be produced in these rats with the diet employed, a second experiment with albino rats of the Wistar strain, supplied by Glaxo Laboratories, Ltd., was carried out. In the later experiments both black and white and albino rats bred in this Institute were used. It is not certain whether the strain of albino rats bred in this Institute is identical with that obtained from Glaxo Laboratories. In the first two experimental series littermates were distributed in the different test groups; the weights of the animals at the beginning of the experiments were between 75 and 80 g. and their ages varied between 40 and 45 days. In the later experiments the animals used were not necessarily litter mates and their weights and ages varied more widely.

The animals were fed and housed separately; they were kept during most of the time in metabolism cages of the usual design allowing separation of urine and faeces. The receptacles for the urine contained a small quantity of 0·01 N-HCl and toluene to prevent, as far as possible, oxidation of homogentisic acid. The volume of the urine was usually very small and the cages were washed with a constant amount of 0·01 N-HCl; urine and cage washings were combined and made up to 25 ml. The diluted urine was then filtered and portions used for the various estimations. In spite of the considerable care taken in the collection of the urines, it is believed that the recovery of the urine was never quantitative and the figures for the daily excretion of homogentisic acid given in this paper are likely to be too low. The rats were weighed three times a week and food consumption was also recorded.

Diets. The amino-acid mixtures used in the first two experimental series were very similar to those described by Glynn *et al.* (1945). In some of the experiments 40 mg. of *l*-phenylalanine/day was given instead of 80 mg. of the racemic compound; neither the onset nor the degree of alcaptonuria was affected by this substitution; this result agrees with the findings of Lanyar (1942) that *d*-phenylalanine produces little homogentisic acid in white rats. In the experiments of Glynn *et al.* (1945) 75 mg. of $NaHCO_3$/day was administered in order to neutralize the HCl derived from the hydrochlorides of the basic amino-acids supplied. This produced a neutral or even slightly alkaline urine leading to oxidation of homogentisic acid. Three of the rats were, therefore, given the basal diet without any bicarbonate, whilst the other two received the same diet as that used in the earlier work. The five rats developed alcaptonuria on the same day and the bicarbonate supplement was therefore omitted from the diets. The basal diet contained neither methionine nor cystine, whilst the cystine-supplemented diet supplied 30 mg. of that amino-acid/day. The vitamin supplements were identical with those used by Glynn *et al.* (1945), save that vitamin E was supplied in the form of α-tocopherol acetate (0·1 ml. of a 1% solution/week). The carbohydrate-salt mixture was the same as that used in the earlier work. The 'non-protein' diet was identical with the other diets except that the amino-acid mixture was replaced by an equivalent amount of starch. Several rats were given 'tryptophan-deficient' and 'lysine-deficient' diets; the former received 1 g. of zein supplemented with 0·1 g. of lysine hydrochloride and the latter 1 g. of zein and 0·03 g. of *l*-tryptophan/day. It was calculated that these diets provided about 0·05 g. of tyrosine and 0·06 g. of phenylalanine daily. The non-protein part of the diet was the same as that given to the other groups. All supplements were incorporated in the basal diet. As in the earlier experiments the intake of amino-acids or protein and of the vitamins was kept constant and the food intake was adjusted to the appetite by varying the amount of the carbohydrate-salt mixture supplied.

Chemical methods. The methods for the estimation of homogentisic acid are discussed by Neuberger (1947) and only a few details need be mentioned here. The alkali test was used in a qualitative manner and all the data given in this paper were obtained by the iodometric method. However, the molybdate method was also applied to most urine samples and gave generally good agreement with the iodometric titration. In some urines the values obtained with the colorimetric method were very much higher than those indicated by titration. This discrepancy was shown to be due to the presence of *p*-hydroxyphenylpyruvic acid which reduces the molybdate reagent but is not reversibly oxidized by iodine. Such urine specimens contained substances soluble in ether and giving a positive reaction with the Millon reagent, whilst no such substances were found in all other urines for which concordant results were obtained by the iodometric and molybdate methods respectively.

An approximate estimation of *p*-hydroxyphenylpyruvic acid was carried out in the following manner. To 5 ml. of diluted acidified urine were added 2·5 ml. of a 20% NaCl solution containing 10% HCl. This solution was extracted three times with ethyl acetate, 5 ml. being used for each extraction. The combined extracts were washed once with 15 ml. of 0·02 N-HCl containing 20% NaCl and the keto acid was then extracted with three lots of 5 ml. each of a 0·42% $NaHCO_3$ solution. The bicarbonate solutions were then combined and a portion was used for the estimation of the keto acid by the Millon method as modified by Medes (1932) and by the method of Penrose & Quastel (1937) which estimates keto acids. The results thus obtained by the two techniques agreed well; the values were also similar to those calculated from the differences of the results between the iodometric and colorimetric estimations on the urine directly.

RESULTS

The effects of feeding amino-acid mixtures deficient in sulphur to black and white rats. Five black and white rats belonging to the same litter were divided into two groups; three animals were given a diet deficient in both cystine and methionine, whilst two rats received an amino-acid mixture lacking only methionine. A sixth litter-mate was given the basal amino-acid diet supplemented by 0·08 g. *dl*-methionine/day. Animals in both deficient groups lost weight at a fairly similar rate which averaged over the whole period about 0·75–0·95 g./day. The weight curves fell steeply during the first week of the deficiency and then flattened out. No trace of homogentisic acid was excreted by any of the animals; on the 17th day of the experiment 40 mg. of additional *l*-phenylalanine was given to the sulphur-deficient rats and this supplement was administered for three

successive days. No alcaptonuria developed. After 42–45 days the animals were killed; the rats on the deficient diets which had been weighing between 80 and 83 g. at the beginning of the experiment had by then lost between 37 and 41 g. Their organs, particularly the livers, were examined both macroscopically and histologically. There was no evidence of necrosis. The one rat on the diet containing methionine grew at a rate of about 2 g./day; it was killed after 30 days and no abnormalities were found.

The effects of cystine and methionine deficiency on albino rats

General symptoms. Since the earlier experiments of Glynn *et al.* (1945), in which alcaptonuria and necrosis were observed were done on albino rats, it was thought desirable to repeat this work with rats of that strain. Wistar rats (Glaxo Laboratories, Ltd.), all litter-mates, were therefore divided into two groups; animals G 1, G 2, G 4, G 5 and G 8 received the basal diet only which was almost free of methionine and cystine, whilst the animals G 6 and G 7 received the basal diet supplemented with cystine (30 mg./day). The animals in both groups lost weight rapidly (Table 1), though it appeared that

Table 1. *Weight changes and date of appearance of alcaptonuria of rats receiving a diet deficient in both cystine and methionine (A) and of rats receiving a diet deficient in methionine only (B)*

No. and sex of rat	Diet	Weight at the beginning of the experiment (g.)	Loss of weight during first 30 days (g.)	First appearance of homogentisic acid in the urine (days)
G 1 ♂	A	76	29	14
G 2 ♀	A	75	30	12
G 4 ♂	A	78	31	15
G 5 ♀	A	79	31	12
G 8 ♂	A	77	27	11
G 6 ♂	B	77	22	—
G 7 ♀	B	75	24	—

the rate of this decrease in weight was slower in the rats receiving cystine than in the other animals reared on the basal diet alone. During the first two weeks of the experiments there were no obvious signs of nutritional deficiency, apart from loss of weight. Later, however, the animals lost their hair and during the last days of the experiment many of the animals were ill and some were killed when obviously dying. Apart from the symptoms of wasting, such as almost complete disappearance of depot fat, no gross abnormalities were found. There was no definite evidence of necrosis of the liver in any of the animals. Parts of the livers of the animals used in this and the first experiment were taken for the estimation of glutathione. The results of these determinations are reported in a separate paper (Leaf & Neuberger, 1947).

Onset of alcaptonuria. This occurred at almost exactly the same time in all the five animals of the first group (Table 1). The daily excretion of homogentisic acid on the basal diet alone varied considerably from day to day even in the same animal. It

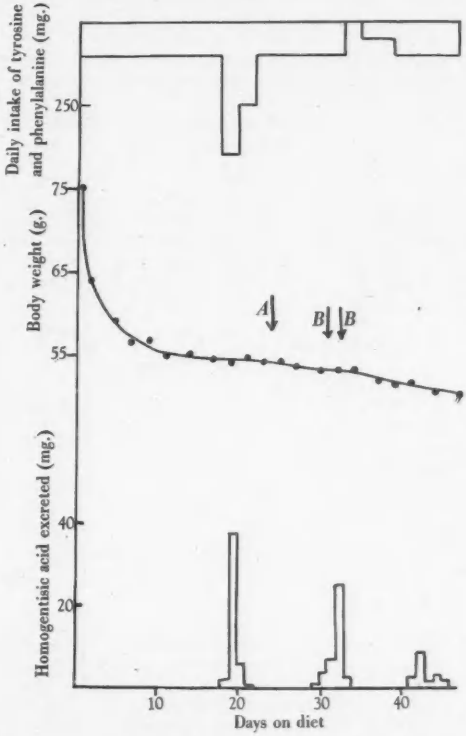

Fig. 1. The effect of cystine on the excretion of homogentisic acid of rat G 7. The animal received 0·03 g. *l*-cystine/day from the beginning of the experiment. On the 24th day ($A\downarrow$) cystine was left out of the diet. On the 31st and 32nd day 0·06 g. homogentisic acid/day was given ($B\downarrow$). The supplements to the basal diet indicated in the top of the graph consisted of *l*-tyrosine.

was usually between 5 and 10 mg., but values as low as 0·5 mg. and as high as 15 mg. were occasionally found. When no extra aromatic amino-acids were fed, it was found that only about 6–10% of the tyrosine and phenylalanine present in the diet were converted to homogentisic acid and this proportion did not increase significantly during the experiment. Complete removal of either tyrosine or phenylalanine from the diet led to a complete disappearance of the alcaptonuria (Figs. 1 and 2). Increasing

the intake of phenylalanine or tyrosine led to a considerable absolute and relative rise of excretion of homogentisic acid. With higher levels of intake, particularly of *l*-phenylalanine, the rate of conversion to homogentisic acid approached almost 50 % (Fig. 3).

Effect of the administration of cystine on alcaptonuria. When cystine was given from the beginning of the experiment no alcaptonuria developed (Figs. 1 and 2) unless the intake of aromatic amino-acids was substantially raised. These findings agree with those reported by Glynn *et al.* (1945) who did not find any evidence of alcaptonuria in rats receiving cystine but no methionine for periods of up to 147 days. When rats G 6 and G 7 which had been on the methionine-deficient diet for 19 days, were given 0·3 g. of extra tyrosine for three successive days, they promptly excreted homogentisic acid (Figs. 1 and 2). Reduction of the tyrosine intake produced a corresponding decrease in the excretion of homogentisic acid and the alcaptonuria again disappeared, when the animals were returned to the basal diet containing 100 mg. of aromatic amino-acids (Figs. 1 and 2). Removal of cystine from this diet produced alcaptonuria within a week, although the intake of tyrosine and phenylalanine had not been increased during that period (Figs. 1 and 2).

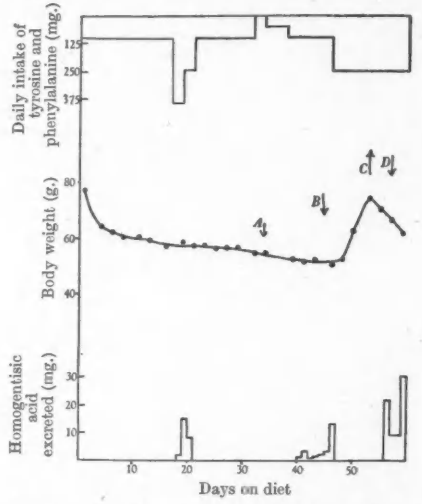

Fig. 2. The effect of cystine and methionine on the excretion of homogentisic acid of rat G 6. This rat received 0·03 g. *l*-cystine/day from the beginning of the experiment; $A\downarrow$ indicates termination of cystine supplement. $B\downarrow$ indicates addition of 0·08 g. *dl*-methionine/day. This was again removed from the diet after 6 days ($C\uparrow$). *l*-Cystine (0·08 g./day) was added 4 days later ($D\downarrow$). Amino-acid supplement consisted of *l*-tyrosine.

The administration of cystine to rats which had developed alcaptonuria on the cystine-free basal diet produced equivocal results. In several experiments the excretion of homogentisic acid appeared to be significantly reduced (Fig. 4), in others no definite change was observed (Fig. 5). Similar results were obtained with cysteine (Figs. 4 and 5).

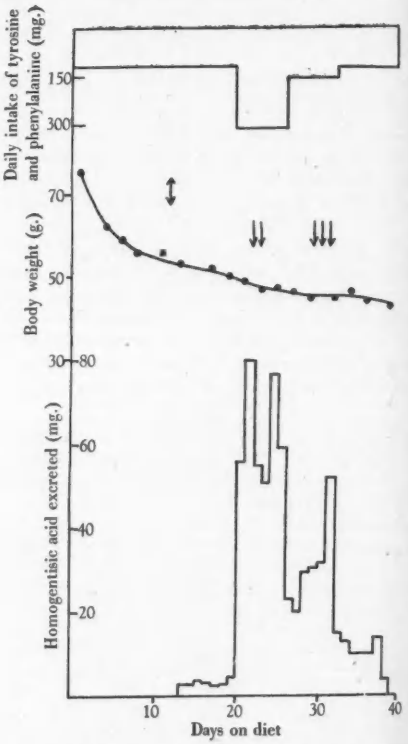

Fig. 3. The effect of ascorbic acid and *l*-phenylalanine on the excretion of homogentisic acid of rat G 2. This rat received no cystine in its basal diet. The variations in the intake of aromatic amino-acids were produced by additions of *l*-phenylalanine. The double-headed arrow indicates the first appearance of homogentisic acid in the urine. All other arrows indicate administration of *l*-ascorbic acid (0·03 g./day). Supplements consisted of *l*-tyrosine.

Effect of the administration of methionine on alcaptonuria. When 0·08 g. of *dl*-methionine/day was given to animals which had been on the basal diet alone or on the basal diet supplemented with cystine, resumption of growth occurred in a dramatic fashion. The animals gained about 4 g./day and doubled their weight in about 12 days. Alcaptonuria disappeared within 24 hr. of the giving of methionine

(Fig. 5) and even a greatly increased intake of tyrosine was well tolerated. Indeed, a total daily intake of 0·5 g. of aromatic amino-acids failed to produce alcaptonuria. When after a period of methionine administration this amino-acid was again removed from the diet, alcaptonuria reappeared after an interval of a few days, i.e. much sooner than was found at the beginning of the experiment (Fig. 2).

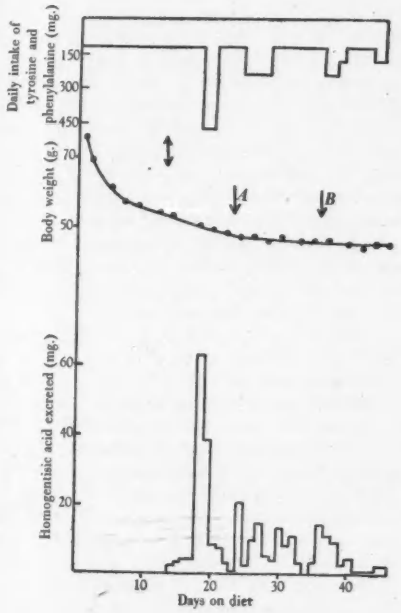

Fig. 4. The effect of cystine and cysteine on the excretion of homogentisic acid of rat G 4. This rat had received no cystine in its basal diet. $A\downarrow$ indicates administration of *l*-cystine (0·06 g./day). Later ($B\downarrow$) this was replaced by *l*-cysteine hydrochloride (0·1 g./day). The double-headed arrow indicates onset of alcaptonuria. Amino-acid supplements consisted of *l*-tyrosine.

Effect of ascorbic acid on alcaptonuria. Since scorbutic guinea-pigs excrete homogentisic acid in addition to *p*-hydroxyphenylpyruvic acid in the urine on administration of tyrosine, it was thought possible that the alcaptonuria observed in methionine and cystine-deficient rats might be caused by an interference with the synthesis of this vitamin by the amino-acid deficiency. Figs. 3 and 6 show that administration of ascorbic acid has no demonstrable effect on the excretion of homogentisic acid.

Effect of aromatic keto acids on the output of homogentisic acid. It has already been pointed out that addition of tyrosine and phenylalanine increases homogentisic acid excretion; the corresponding keto acids were found to have similar effects (Fig. 6).

Isolation of homogentisic acid. Acidified, diluted rat urine (300 ml.) estimated to contain about 0·9 g. of homogentisic acid, was extracted with ether in a liquid extractor for 48 hr. The dried ether was concentrated to about 10 ml. and extracted with 30 ml. of water in three equal portions; lead acetate trihydrate (5 g.) dissolved in water (25 ml.) was

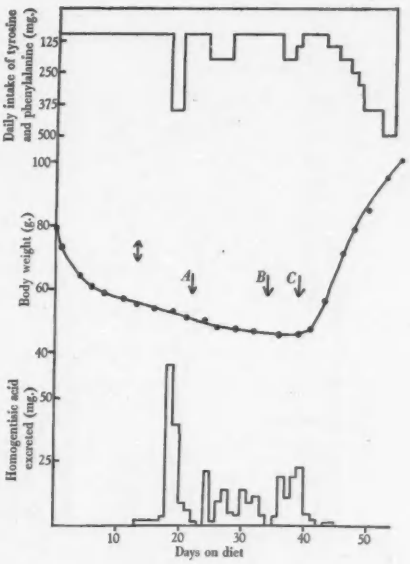

Fig. 5. Comparison of the effect of cystine, cysteine and methionine on the output of homogentisic acid in a rat (G 4) which had no cystine in its basal diet. The double-headed arrow indicates onset of alcaptonuria. Arrows marked *A*, *B* and *C* indicate periods of administration of *l*-cystine (0·06 g./day), *l*-cysteine (0·1 g./day) and *dl*-methionine (0·08 g./day) respectively. Variations of intake of aromatic amino-acids were produced by administration of *l*-tyrosine.

then added at about 80°. The material which crystallized on cooling was filtered off, recrystallized from hot water and air-dried. It amounted to 0·95 g. and had m.p. 215°. (Found: C, 32·0; H, 3·2; PbO, 37·5. Calc. for $C_{18}H_{14}O_8Pb.3H_2O$: C, 32·3; H, 3·36; PbO, 37·5%.) Of the material 120 mg. were dried *in vacuo* over P_2O_5 at 80°; loss of weight found was 10·7 mg. (calc. for the trihydrate 10·9 mg.). The properties agree fully with those described for the lead salt of homogentisic acid by Wolkow & Baumann (1891). The lead salt (300 mg.) was converted into free homogentisic acid by H_2S; the acid was recrystallized from ether and chloroform and had m.p. 146°. There was no depression of m.p., when a sample of this material was mixed with a sample of synthetic homogentisic acid.

Excretion of p-*hydroxyphenylpyruvic acid.* Administration of large doses of extra tyrosine to some of the rats on sulphur-deficient diets gave rise to excretion of *p*-hydroxyphenylpyruvic acid (Figs. 4 and 5). Thus, when rat G 1 received 0·3 g. of extra

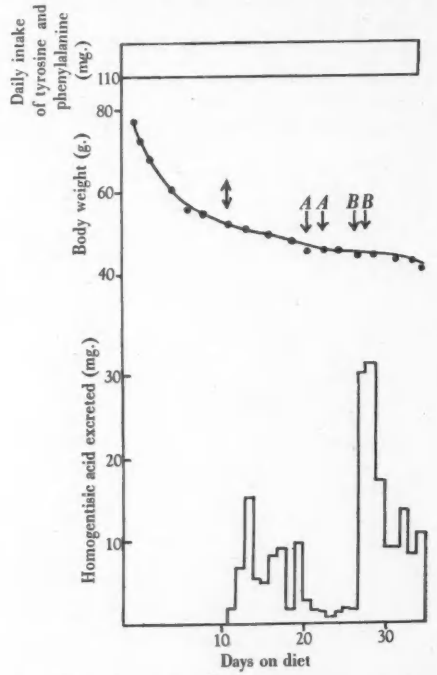

Fig. 6. Effect of ascorbic acid and phenylpyruvic acid on the output of homogentisic acid of rat G 8. The double-headed arrow indicates onset of alcaptonuria. Arrows marked *A* indicate administration of *l*-ascorbic acid (0·01 g./day), whilst arrows marked *B* indicate administration of phenylpyruvic acid (0·05 g./day).

tyrosine on two successive days, the differences between the iodometric and colorimetric values for homogentisic acid indicated amounts of the keto acids of 21 and 32 mg. respectively. Estimations by the Millon method gave values for the two days of 21 and 29 mg. The acid was not isolated, but there is little doubt that it was *p*-hydroxyphenylpyruvic acid, for the following reasons. The substance was found to be acidic and was soluble in water, ether and ethyl acetate. It reduced molybdate in acid solution, but did not react reversibly with iodine. The acid also reacted both with Millon's reagent and 2:4-dinitrophenylhydrazine. The fact that similar values were obtained by three different methods of estimation indicated the absence of hydroxyphenyllactic acid.

Production of alcaptonuria in albino and black and white rats by a protein-deficient diet. The experiments so far described showed that albino rats kept on a diet deficient in methionine and cystine excreted homogentisic acid, though the intake of aromatic amino-acids was low. The question arose whether this increased susceptibility to alcaptonuria could also be induced by a diet deficient in protein. Six rats, three of the albino strain of the Institute, and three of the black and white strain, with initial weights of 85–101 g., were put on the protein-deficient diet. After 3 weeks their weights had dropped to about 70 g.; they were then given varying amounts of tyrosine, but no other amino-acids. After an interval of 1 or 2 days rats of both strains excreted homogentisic acid. With a daily intake of 0·3 g. of tyrosine the daily output of homogentisic acid varied between 30 and 80 mg.; when the daily dose of tyrosine was reduced to 0·2 g. homogentisic acid was between 6 and 25 mg./day and a further reduction of tyrosine to 0·1 g. abolished alcaptonuria altogether in most rats. There was no obvious difference between the two strains. Addition of 0·08 g. of *dl*-methionine or of 0·06 g. of *l*-cystine to the daily ration had no significant effect on the degree of alcaptonuria observed.

Alcaptonuria and deficiency of lysine and tryptophan. Albino rats weighing between 70 and 80 g. were put on zein diets supplemented with lysine hydrochloride and tryptophan respectively. After 3 weeks on the deficient diet they had lost between 15 and 20 g.; they did not excrete any homogentisic acid. Various quantities of tyrosine were now added to their diets and the results of two typical experiments are shown (Tables 2 and 3). It was found that lysine-deficient rats did not excrete homogentisic acid in significant amounts, unless their intake of additional tyrosine, apart from that contained in the basal diet, was about 0·1 g./day. The tryptophan-deficient rats showed alcaptonuria already with a dose of additional tyrosine of 0·05 g. With 0·2 g. of tyrosine the alcaptonuria was very marked in all deficient animals. Neither the addition of cystine nor that of methionine had any effect on the output of homogentisic acid. Supplementation of the lysine and tryptophan-deficient diets with the lacking amino-acid abolished the alcaptonuria in a few days, if the intake of extra tyrosine was kept at the level of 0·15 g. or less. With larger doses of tyrosine, however, the effect of the supplementation was less marked, at least during the comparatively short period of 6 days employed. It is likely that this was due to the fact that zein, even if it is supplemented with lysine and tryptophan, is still quantitatively deficient in several essential amino-acids. When zein was replaced by casein, the alcaptonuria disappeared even with an extra tyrosine intake of 0·3 g./day.

Table 2. *Lysine deficiency and alcaptonuria*

(The two albino rats weighed 70 and 68 g. respectively at the beginning of the experiment. They had been on the deficient diet for 24 days without showing alcaptonuria. Additional tyrosine was then given.)

	Date	Supplement	Homogentisic acid excreted (mg.)
Rat AL 4 (1) ♂	14. vii	0·05 g. *l*-tyrosine	0
	15. vii	"	0
	16. vii	"	0
	17. vii	0·1 g. *l*-tyrosine	5·0
	18. vii	"	0·5
	19. vii	0·15 g. *l*-tyrosine	4·5
	20. vii	"	3·5
	21. vii	0·2 g. *l*-tyrosine	17·0
	22. vii	"	19·0
	23. vii	0·2 g. *l*-tyrosine + 0·075 g. *l*-lysine HCl	40·0
	24. vii	" "	18·0
	25. vii	" "	0
Rat AL 4 (2) ♂	14. vii	0·1 g. *l*-tyrosine	0
	15. vii	"	8·4
	16. vii	"	19·3
	17. vii	"	4·3
	18. vii	"	7·5
	19. vii	0·15 g. *l*-tyrosine	35·9
	22. vii	0·2 g. *l*-tyrosine + 0·1 g. *l*-cystine	83·2
	23. vii	" "	63·0
	24. vii	" "	82·0

Table 3. *Tryptophan deficiency and alcaptonuria*

(The two albino rats had been on the tryptophan-deficient diet for 21 days before tyrosine was given. Their initial weights were 86 and 83 g. respectively.)

	Date	Supplement	Homogentisic acid excreted (mg.)
Rat AL 5 (1)	14. vi	0·05 g. *l*-tyrosine	33·0
	15. vi	"	20·0
	16. vi	"	7·0
	17. vi	0·1 g. *l*-tyrosine	40·0
	18. vi	0·1 g. *l*-tyrosine + 0·025 g. *l*-tryptophan	37·2
	19. vi	" "	28·8
	20. vi	" "	26·7
	21. vi	" "	0
	22. vi	" "	0
Rat AL 5 (6)	14. vi	0·1 g. *l*-tyrosine	10·6
	15. vi	"	24·0
	16. bi	"	30·0
	17. vi	"	42·2
	18. vi	0·1 g. *l*-tyrosine + 0·1 g. *l*-cystine	26·7
	19. vi	" "	35·7
	20. vi	" "	74·0

DISCUSSION

The isolation of homogentisic acid from the urine of albino rats receiving a diet deficient in both cystine and methionine shows that the blackening of the urine observed in the experiments of Glynn *et al.* (1945) was due to alcaptonuria. It is of some interest that excretion of homogentisic acid could be consistently produced in animals which obtained only 65 mg. of tyrosine and 40 mg. of phenylalanine/day, amounts which would be present in any normal diet supplied to rats of that age. It was found that only a small fraction of the ingested aromatic amino-acids was excreted in the urine in the form of homogentisic acid and this proportion increased greatly with rising intake of tyrosine or phenylalanine. Unlike congenital human alcaptonuria, the metabolic lesion produced in rats by this amino-acid deficiency does not involve a complete blockage of the intermediary metabolism of the two aromatic amino-acids. With moderate intakes of tyrosine, homogentisic acid appeared to be the only aromatic metabolite present in the urine in abnormal quantities; only when large amounts of tyrosine were administered was *p*-hydroxyphenylpyruvic acid excreted in measurable amounts. This type of alcaptonuria produced in rats

differs, therefore, from the metabolic disturbances described in premature infants (Levine, Marples & Gordon, 1941) and those occurring in guinea-pigs (Sealock & Silberstein, 1940). Both these conditions are caused by a dietary deficiency in vitamin C and are associated with the excretion of large amounts of *p*-hydroxyphenylpyruvic acid. The alcaptonuria described here is not affected by the administration of ascorbic acid and the differences in excretion products observed may be either due to the fact that various species behave in a different manner or that the metabolic lesion involved varies with the dietary deficiency producing it.

No alcaptonuria could be produced in black and white rats with the sulphur-deficient diet. This might indicate genetic differences between the two strains. However, with a protein-deficient diet and a large intake of tyrosine, black and white rats excreted homogentisic acid in amounts not significantly smaller than those found with albino rats. It is concluded therefore that such a difference between the two strains, if it really exists, is only relative.

When this alcaptonuria was first discovered, it was thought to be due specifically to a deficiency in sulphur-containing amino-acid. This interpretation, however, is not borne out by the present experiments. Alcaptonuria can be produced in white rats receiving a normal diet, if large quantities of *l*-phenylalanine are administered. This was first shown by Papageorge & Lewis (1938) and has been confirmed by various workers (Butts, Dunn & Hallman, 1938; Closs & Braaten, 1941; Abbott & Salmon, 1943; Lanyar, 1943). *l*-Tyrosine has also been shown effective in this respect (Butts, Sinnhuber & Dunn, 1941) and this has been confirmed by other workers. Strangely enough Martin & Hueper (1943) found that their rats, which were fed on diets containing large quantities of tyrosine for several weeks, did not excrete homogentisic acid. This type of alcaptonuria has generally been found not to appear at once and the interval between the administration of the 'extra' amino-acid and the appearance of alcaptonuria is reduced as the dose is increased. It is possible that the inability of the body to metabolize the aromatic amino-acids in a normal manner is, at least partly, caused by the toxic effect of excessive tyrosine, which is either supplied as such or produced in the body from the administered phenylalanine. The dose necessary to produce alcaptonuria was found by most workers to be about 0·4–0·5 g. of amino-acid/100 g. of body weight; or, if the amino-acids given in the basal diet are included, about 0·6 g. Our own unpublished observations also indicate that it is necessary to supply at least 0·65 g. of aromatic amino-acids/100 g. of body weight in order to produce alcaptonuria within a week in the majority of animals given a diet containing 15% of casein. Whether such a linear correlation between minimal dose and body weight really exists is uncertain, and in view of the considerable individual variations in animals a very large number of experiments would have to be done before this point could be considered established. However, with normal animals of very different weights, we have never been able to produce alcaptonuria unless the total intake of aromatic amino-acids was at least 0·5 g./100 g. of body weight and this agrees with the findings of other workers.

The alcaptonuria observed in sulphur-deficient rats already appears, where the total intake of the two aromatic amino-acids is about 0·1 g./day or 0·15 g./100 g. of body weight. It follows therefore that the decreased capacity of these animals to metabolize tyrosine and phenylalanine in a normal manner, can only partially be explained by the reduction in body weight associated with the dietary deficiency. This is also clearly shown in the experiments in which addition of methionine to a sulphur-deficient diet raised the tolerance to tyrosine quite out of proportion to the increase in weight. It appears that even if the change of weight is taken into account, deficiency of methionine and cystine reduces the minimal dose of aromatic amino-acids necessary to produce alcaptonuria to about a fourth of that found with animals on a normal diet. The results reported here also indicate that this effect is not specifically associated with a deficiency of one particular amino-acid. Thus the administration of cystine or methionine to rats receiving 200 mg. of tyrosine in addition to a protein-deficient diet, does not reduce the excretion of homogentisic acid observed under such conditions. Moreover, the observation that the addition of 50 or 100 mg. of tyrosine to diets deficient in tryptophan and lysine respectively produces alcaptonuria, suggests that any deficiency of an essential amino-acid leads to a decreased ability of the animal to break down aromatic amino-acids in a normal manner. The total intake of aromatic amino-acids/100 g. of body weight for animals just showing alcaptonuria was about 220 mg. for tryptophan deficiency and about 300 mg. for lysine deficiency. All these observations are compatible with the assumption that any deficiency of an essential amino-acid reduces the level of intake of tyrosine and phenylalanine necessary to produce alcaptonuria, and that the differences observed with the various diets are those of degree rather than of kind. When both methionine and cystine are lacking in the diet, alcaptonuria occurs even when only 'normal' amounts of tyrosine and phenylalanine are present in the diet. If cystine is supplied and the deficiency is confined to methionine, the intake of aromatic amino-acids has to be somewhat increased, before homogentisic acid is excreted; a deficiency of tryptophan appears to have similar effects to that of methionine, whilst lack of lysine

interferes with the metabolism of aromatic amino-acids to a lesser extent. Whether any deficiency of an essential metabolite will have similar effects must remain a matter for further investigation.

Closs & Braaten (1941) in their investigations on the metabolism of large doses of *l*-phenylalanine in rats, found that the nature of the excretion product was influenced by the acid-base balance of the organism and that homogentisic acid is only excreted if alkali is administered together with the amino-acid. A somewhat similar effect on the reduction of the output of homogentisic acid in scorbutic guinea pigs by acidic substances was reported by Sealock (1942). We have therefore given some attention to the pH of the urines of the rats on amino-acid diets. Alcaptonuria was observed at about the same time in animals receiving bicarbonate and excreting a neutral or alkaline urine as in animals which had not been supplied with bicarbonate to neutralize amino-acid hydrochlorides, and which excreted a urine of a pH of about 4·8. As reported above, in the later phases of the experiment no alkali was given and it is concluded therefore that alkalosis need not be considered in the interpretation of these results.

The sulphur-deficient diets employed in this work have been used by Glynn *et al.* (1945) to produce massive hepatic necrosis, and the failure to repeat these results calls for an explanation. It is probable that animals on a diet completely deficient in methionine and cystine are less susceptible to liver damage than rats receiving very small amounts of a sulphur-containing amino-acid; this is suggested by the results of Himsworth & Glynn (1944) who found that diets containing 4–6 % of casein are more likely to produce necrosis than diets with no protein or diets containing only 2 % of casein. It was also found (Glynn *et al.* 1945) that necrosis was more easily produced in rats given 8 mg. of methionine/day than in animals receiving no amino-acid sulphur. Moreover, the animals on the sulphur-deficient diet used in this work lost weight very rapidly and appeared to die of inanition before necrosis could develop. It is also possible that the tocopherol which was given in our experiments, but not by Glynn *et al.* (1945) had some protective effect; this is suggested by the results of Schwarz (1944) who found that tocopherol protected rats against liver necrosis produced by an unknown factor. Data at present available do not permit us to decide which of these explanations is correct and it can only be stated that, in spite of the large amount of work done on this subject, we are still ignorant of the exact conditions required for the production of hepatic necrosis and exactly how the diet affects and facilitates liver damage.

SUMMARY

1. Homogentisic acid has been isolated from the urine of rats fed a diet deficient in methionine and cystine and containing only relatively small amounts of tyrosine and phenylalanine. If cystine was added to such a diet, no alcaptonuria occurred, unless the tyrosine intake was also raised. When large amounts of tyrosine were administered, some *p*-hydroxyphenylpyruvic acid was also excreted.

2. Alcaptonuria was also produced if tyrosine was added to a protein-deficient diet. The excretion of homogentisic acid occurring under these conditions was not affected by the administration of cystine or methionine.

3. It was found that the threshold of the intake of phenylalanine and tyrosine necessary to produce alcaptonuria was significantly reduced by a deficiency of tryptophan and, to a lesser extent, by a lack of lysine. It is suggested that any deficiency of an essential amino-acid may impair the capacity of the organism to deal in a normal manner with the two aromatic amino-acids.

4. No hepatic lesions were found in any of the animals. Possible reasons for such negative findings are discussed.

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Studies in the Biochemistry of Micro-organisms

74. THE MOLECULAR CONSTITUTION OF GEODIN AND ERDIN, TWO CHLORINE-CONTAINING METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM. PART 3. POSSIBLE STRUCTURAL FORMULAE FOR GEODIN AND ERDIN

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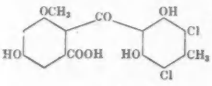
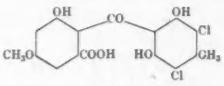
Geodin and erdin were first described by Raistrick & Smith (1936) and further characterized by Clutterbuck, Koerber & Raistrick (1937). They are two chlorine-containing metabolic products of the mould *Aspergillus terreus* Thom of empirical formulae $C_{17}H_{12}O_7Cl_2$ and $C_{16}H_{10}O_7Cl_2$ respectively. Calam, Clutterbuck, Oxford & Raistrick (1939) later synthesized the trimethyl ethers of the reduction products of these substances, showed that dihydrogeodin is the methyl ester of dihydroerdin, and proved that the latter has one of the two following structural formulae:



I

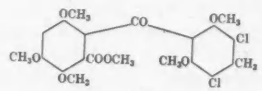


II

Of these the structure I is perhaps more likely to be correct since dihydrogeodin does not quickly give a blue coloration with 2:6-dichloroquinone-chloroimide. We are indebted to Prof. A. R. Todd for drawing our attention to this reaction which, if positive, indicates the presence of a free position *para* to a phenolic group (cf. Gibbs, 1927). Although when an immediate blue colour is given in this reaction it is usually a reliable guide it may occasionally give positive results when the *para* position is occupied (cf. Davidson, Keane & Nolan, 1943) and we have also encountered heavily substituted phenols which give a negative reaction when the *para* position is free. Examples of this are the mould metabolic products 3:5-dihydroxy-2-carboxybenzoyl methyl ketone and the corresponding carbinol (Oxford & Raistrick, 1933). 3:5-Dihydroxyphthalic acid also gives a quite atypical purplish brown colour with the reagent. Gibbs himself stated that the reaction failed in certain unspecified instances so that structure II for dihydroerdin cannot be ruled out by the negative indophenol reaction alone.

Before discussing the structures of the metabolic products themselves it will be convenient to describe some further degradation products of geodin and erdin.

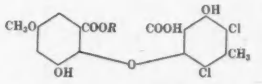
(*a*) *Methylated geodin.* Clutterbuck *et al.* (1937) prepared methylated geodin, and the identical methylated erdin, by the action of methyl sulphate and alkali on the parent compounds. In the formation of methylated geodin a molecule of water is added and the resultant product contains a total of six methoxyl groups. It has now been shown that this compound has the structure III since on treat-



III

ment with 80% sulphuric acid (cf. Graebe & Eichengrun, 1892) it yielded 2:6-dichloro-3:5-dimethoxy-*para*-orsellinic acid and a dimethoxyhydroxybenzoic acid which on methylation gave the known 2:3:5-trimethoxybenzoic acid.

(*b*) *Geodin and erdin hydrates.* Corresponding products from erdin and geodin were obtained by treatment of the parent compounds with 80% sulphuric acid. Under these conditions there are obtained the dibasic *erdin hydrate* and its monomethyl ester *geodin hydrate*, identical compounds being obtained therefrom on full methylation or by ethylation followed by hydrolysis. These compounds give a violet colour with ferric chloride and a blue colour with 2:6-dichloroquinone-chloroimide, and their structures are represented almost certainly by IV, for the following reasons.



IV. Erdin hydrate: R=H. Geodin hydrate: R=CH_3.

Fully methylated geodin hydrate has the molecular formula $C_{20}H_{20}O_9Cl_2$; twelve of the carbon atoms are accounted for by the two benzene rings,

two by carboxyl groups, five by methoxyl groups and one by a methyl side chain. The above groups also account for all twenty hydrogen atoms and seven oxygen atoms, while the two chlorine atoms will be attached to the benzene ring in the usual way. The remaining atom is a single oxygen which can reasonably be placed only as a bridge between the two rings. The structure IV is in accordance with the colour reactions mentioned above.

(c) *Miscellaneous reactions.* As was pointed out by Raistrick & Smith (1936) geodin is optically active while erdin is not. As there was a possibility that erdin might have been racemized during isolation two attempts were made to resolve it, using brucine and *d*-α-phenylethylamine. Both attempts were unsuccessful and it was concluded that erdin probably contains no asymmetric centre.

Although the carbonyl groups of the dihydro derivatives of geodin and erdin are not reactive geodin readily forms an oxime. This reaction shows that geodin, and presumably erdin, probably contain a second keto group. From this and the fact that geodin and erdin form addition compounds with diazomethane (Raistrick & Smith, 1936) it may be inferred that, although almost colourless, they may still contain a quinonoid or potential quinonoid structure in the non-chlorinated ring. The absorption spectra of geodin, erdin and their dihydro derivatives are not incompatible with the presence of such a structure. In alcoholic solution their main features are strong absorption at the shorter wavelengths followed by a peak at 275–285 mμ. After this the absorption falls off to a low value at about 360 mμ. There is no evidence of a peak in the visible region that would be expected if a true quinone were present.

The actual peaks fell thus:

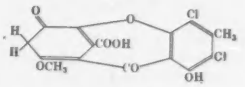
geodin	284 mμ; Σ 15,000
erdin	274 mμ; Σ 16,000
dihydrogeodin	279 mμ; Σ 20,000
dihydroerdin	285 mμ; Σ 16,000

When dihydrogeodin or dihydroerdin is boiled with hydriodic acid fission of the molecule takes place and α-resorcylic acid and orcinol can be isolated (Calam *et al.* 1939). When geodin or erdin are similarly treated no fission of the molecule occurs, one molecule of carbon dioxide is evolved and from the mother liquor two isomeric non-chlorinated substances can be isolated. These substances, nor*geodin A* and nor*geodin B* each have the composition $C_{14}H_{10}O_5 . H_2O$ and on treatment with diazomethane, di-ethers, and with dimethyl sulphate tri-ethers, of $C_{14}H_{10}O_5$ in each instance are produced. The structure of *nor*geodin B is obscure, but the yellow *nor*geodin A is almost certainly a xanthone since its trimethyl ether was soluble in conc. HCl and readily formed a ferrichloride.

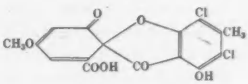
DISCUSSION

Constitution of geodin and erdin

The above evidence is sufficient to limit the reasonable structures for erdin to two only. The formation of the hydrates (IV) with sulphuric acid and the methylation product (III) obtained with dimethyl sulphate and alkali indicate the presence of an oxygen bridge between the two six-carbon rings in addition to the carbonyl group already demonstrated, but afford no certain indication of its point of attachment, relative to the —CO—, to the non-chlorinated ring. The reactions with hydroxylamine and with diazomethane suggest the presence of a quinonoid or potential quinonoid structure. On this basis we suggest for erdin the alternative structures V and VI.



V. Erdin.



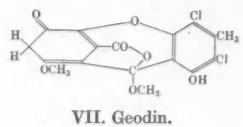
VI. Erdin.

The principal reactions of erdin can be readily explained by these structures. On hydrogenation the oxygen link presumably breaks yielding I and II respectively. On treatment of erdin with dimethyl sulphate and alkali a similar fission appears to occur but with the formation of a new hydroxyl group on the α-resorcylic acid nucleus. With either V or VI this would appear at the 2 position, since in the latter case the OH group might migrate from the point of attachment in accordance with the known reactions of quinonoid systems. On acetylation under mildly acidic conditions a similar reaction presumably occurs analogous to the Thiele acetylation of quinones (cf. Clutterbuck *et al.* 1937). With strong acid, not the —O— but the carbonyl link breaks giving the diphenyl ether IV. This reaction rules out the *para* quinonoid structure corresponding to VI since the product (geodin or erdin hydrate) gives a positive reaction at once with 2:6-dichloroquinonechloroimide. In the above reactions it is assumed that the non-aromatic structure reverts automatically to the corresponding phenolic structure when one of the links between the rings is broken. Either structure will serve to explain the formation of a xanthone, *nor*geodin A, by rearrangement after reductive decarboxylation.

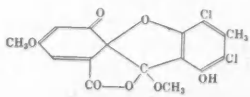
In criticism of these structures it may be said of (V) that, although it readily explains the chemical reactions of erdin, and contains no asymmetric carbon atom, there is really nothing to prevent the non-chlorinated ring from changing to the phenolic form with loss of ketonic properties. The seven-membered ring is also unorthodox but this is hardly a valid reason for rejecting it, since a not unduly strained three-dimensional model of structure (V) can be constructed. Structure (VI) should be stable, but is unsatisfactory in so far as the dihydro compound derived from it would be a phenol with a free *para* position and it would have to be assumed that the negative outcome of the indophenol reaction was due to the heavy substitution of the phenolic ring as mentioned earlier. Structure (VI) also contains an asymmetric carbon atom and hence erdin should be resolvable; as mentioned above efforts to effect such a resolution failed. To sum up, on present evidence it seems that erdin must be either (V) or (VI) but there is at the moment no certain means of distinguishing between them.

Geodin appears to be best regarded as one form of the methyl ester of erdin, and we are greatly indebted to Dr C. H. Hassall for suggesting the *pseudo*-ester structures (VII) and (VIII) based on (V) and (VI) respectively. Stable *pseudo*-esters of *o*-benzoylbenzoic acid are known (Meyer, 1904, 1907) and must inevitably contain at least one asymmetric carbon atom; hence on this formulation there is no difficulty in accounting for the optical activity of geodin, even if structure (V) is assumed for erdin. Raistrick & Smith (1936) found that the treatment of geodin and erdin with diazomethane yielded isomeric but not identical products. The assumption that geodin is a *pseudo*-ester provides a satisfactory explanation for this behaviour also.

The possible presence of a *pseudo*-ester grouping in a natural product is of considerable interest because it has been found that while erdin (the γ-keto acid) shows no antibiotic activity, geodin (the supposed *pseudo*-ester) is antagonistic to the growth of many micro-organisms. The results of tests with a number of bacteria are recorded in an appendix to this memoir.



VII. Geodin.



VIII. Geodin.

EXPERIMENTAL

Hydrolytic fissions

(i) *Of geodin tetramethyl ether.* The compound (1·006 g.), prepared as described by Clutterbuck *et al.* (1937) was heated in a stream of nitrogen with water (10 ml.) and conc. H_2SO_4 (20 ml.). At a bath temperature of 150° the colour of the solution changed, the oil bath was removed and the flask allowed to cool. The nitrogen was passed into a bubbler of standard baryta solution, and the CO_2 produced was equivalent to 7·4 ml. 0·1 N-NaOH.

After adding 25 ml. water the flask was cooled and the solid filtered off and ground with saturated $NaHCO_3$ solution. The $NaHCO_3$ insoluble part was purified by subliming in high vacuum and was proved to be 2:6-dichloro-orcinol dimethyl ether by its mixed m.p. of 130–131°. (Yield: 0·08 g.; calc. from CO_2 produced 0·075 g.) The bicarbonate-soluble part was precipitated by acid, and the solid (0·29 g.) purified by sublimation and crystallization from aqueous ethanol; colourless hair-like needles were obtained, m.p. 201°, which were found to be identical with synthetic 2:6-dichloro-3-hydroxy-5-methoxy-*p*-toluic acid (cf. Calam & Oxford, 1939).

The original H_2SO_4 mother liquor was extracted continuously with ether for 6 hr. On evaporation 0·21 g. of crystals remained behind. Purified by subliming in high vacuum they gave an intense royal blue with $FeCl_3$, a negative Millon reaction, a negative halogen test, were acid to litmus and melted at 176–180°. Found: OCH_3, 31·55; a *dimethoxyhydroxybenzoic acid* requires OCH_3, 31·31 %. On treatment with dimethyl sulphate and alkali in acetone under the usual conditions, 2:3:5-trimethoxybenzoic acid was obtained, m.p. 101–102°, identical in all respects with a synthetic sample prepared from 5-hydroxyhemipinic acid as described by Faltis & Kloiber (1929). (Found: C, 56·56, 56·72; H, 5·67, 5·77; OCH_3, 43·35. $C_{10}H_{12}O_5$ requires C, 56·59; H, 5·70; OCH_3, 43·83 %.)

(ii) *Of geodin and erdin. The production of geodin and erdin hydrates.* Geodin (0·5 g.) was heated on the boiling water bath for 5 min. with conc. H_2SO_4 (10 ml.) and water (5 ml.) and then kept overnight. Water (100 ml.) was added and the product filtered and dried, giving a pale yellow solid which was extracted with hot chloroform to remove unchanged geodin. The residue was purified by crystallizing from aqueous ethanol which gave very pale yellow prisms, m.p. 209°. With $FeCl_3$ an intense violet colour was produced and with 2:6-dichloroquinone-chloroimide an almost immediate pure blue colour as with phenol itself. (Found: C, 49·03, 48·82; H, 3·55, 3·58; Cl, 16·9, 16·8; OCH_3, 15·42, 15·3. $C_{17}H_{14}O_8Cl_2$ requires C, 48·96; H, 3·39; Cl, 17·00; OCH_3, 14·87 %.) Erdin, treated in the same way, gave a khaki-coloured solid forming pale brown parallelograms from aqueous ethanol, m.p. 222–223°. With $FeCl_3$ a violet colour is produced. (Found: C, 47·86, 47·87; H, 3·25, 3·20; OCH_3, 8·43. $C_{16}H_{12}O_8Cl_2$ requires C, 47·66; H, 3·00; OCH_3, 7·69 %.)

Methylation

Erdin hydrate (0·53 g.) was shaken with 5 ml. dimethyl sulphate and 10 ml. acetone, and eight successive lots of 5 ml. 2 N-NaOH were added at intervals of 5 min. A glassy solid remained after the liquid had been decanted, and this formed colourless needles from aqueous ethanol,

m.p. 120–121°, giving no colour with $FeCl_3$. (Found: C, 51·90, 52·17; H, 3·94, 4·06; Cl, 15·45; OCH_3, 35·1; CH_3-C (Kuhn-Roth), 2·99. $C_{20}H_{20}O_5Cl_2$ requires C, 52·30; H, 4·39; Cl, 15·44; $(OCH_3)_5$, 33·8; CH_3-C, 3·27%.)

Hydrolysis. The above compound (0·1 g.) was refluxed 1 hr. with ethanol (10 ml.) and N-NaOH (5 ml.). After removing the ethanol *in vacuo* and acidifying, the product (0·07 g.) was crystallized from ethyl acetate/light petroleum when it formed colourless needles, m.p. 239–240°. (Found: OCH_3, 21·09; equivalent, 220. $C_{18}H_{16}O_5Cl_2$ requires $(OCH_3)_3$, 21·57%; equiv. (as a dibasic acid), 215·5.)

Ethylation and hydrolysis of geodin hydrate

Geodin hydrate (0·5 g.) was refluxed during 4½ hr. with sodium (0·12 g.), ethyl iodide (5 ml.) and ethanol (15 ml.), when the solution became neutral. The solvents were removed as far as possible, and the residue hydrolyzed with 2N-NaOH (15 ml.) and ethanol (15 ml.). After distilling off the ethanol, filtering and acidifying, a solid separated (0·38 g.). It formed rosettes of needles from ethyl acetate/light petroleum, m.p. 206–207°. (Found: C, 51·47; H, 4·53; Cl, 15·2; OCH_3, 20·8. $C_{20}H_{20}O_5Cl_2$ requires C, 52·30; H, 4·39; Cl, 15·44; $(OC_2H_5)_2 + OCH_3$, calc. as $(OCH_3)_3$, 20·25%.)

Erdin hydrate was treated in the same way and gave identical rosettes of needles, whose m.p. was not depressed on mixing with the above product from geodin hydrate.

Reaction of geodin and erdin with HI

Geodin (1·0382 g.) was heated in a Zeisel flask with HI (*d*, 1·7) (15 ml.) in a stream of CO_2-free nitrogen, and the gases were passed through ethanolic $AgNO_3$ and standard baryta water. The bath temperature was 140–150°. CO_2 was evolved equivalent to 51·65 ml. 0·1N-NaOH (calc. for 1 CO_2 per mol., 52·0 ml.). From the $AgNO_3$ solution was obtained 1·167 g. AgI (calc. 1·22 g. AgI for 2OCH_3). The contents of the Zeisel flask were poured into water and the solid filtered off (0·68 g., calc. 0·72 g.). This mixture consists of two isomerides, *nor*geodin A and *nor*geodin B, and they can be separated either by precipitating from warm acetone with 2 vols. chloroform, when the less soluble *nor*geodin B crystallizes out; or better, by extracting the soluble A with warm glacial acetic acid. About 10 parts of B are present to 1 of A.

Norgeodin A, crystallized repeatedly from aqueous ethanol, formed bright yellow needles (with 1 mol. H_2O) with a max. m.p. 305° dec., darkening commencing at 275°. It gives an intense green colour with $FeCl_3$. It has no acidic properties. (Found: C, 60·89; H, 4·41. $C_{14}H_{12}O_6$ requires C, 60·87; H, 4·38%.)

Norgeodin B formed pale yellow needles from acetone/chloroform, max. m.p. 325–330° dec., darkening beginning at 300°. It gives no colour with $FeCl_3$, and is free from Cl. (Found: C, 60·76, 60·99; H, 4·27, 4·44. $C_{14}H_{12}O_6$ requires C, 60·87; H, 4·38%.) It is slowly soluble in $NaHCO_3$ solution. Erdin, treated in the same way, gave the same two products as did geodin, their identity being shown by comparing their trimethyl derivatives.

Methylation products

(*a*) *With dimethyl sulphate.* *Nor*geodin A (0·15 g., m.p. 305° dec.) was treated with dimethyl sulphate in acetone and NaOH in the usual way. The trimethyl ether separated out and was filtered off (0·15 g.). From ethanol containing a little water were obtained very pale yellow needles, m.p. 216–217°, which gave no colour with $FeCl_3$. (Found: C, 67·57, 67·52; H, 5·13, 5·31; OCH_3, 30·8, 30·7. $C_{17}H_{16}O_5$ requires C, 68·00; H, 5·37; $(OCH_3)_3$, 30·97%.)

That it is likely that *nor*geodin A is a xanthone is shown by the fact that the above trimethyl ether slowly dissolves in conc. HCl, in the same way as does xanthone itself. On adding $FeCl_3$ to such a solution a red *ferrichloride* separates. On crystallizing from hot glacial acetic acid its m.p. can be raised to 181° (decomp.), but after a few recrystallizations it begins to decompose. (Found: Fe, 10·8, 10·8; calc., 11·2%.)

*Nor*geodin B (0·35 g., giving no colour with $FeCl_3$) was methylated as above. The colourless product which separated formed needles from ethanol, and melted at 198·5–199·5°. The ethanolic solution, like that of *nor*geodin A trimethyl ether, shows a marked bluish fluorescence. (Found: C, 67·79, 67·67; H, 5·17, 5·30; OCH_3, 30·8, 31·3. $C_{17}H_{16}O_5$ requires C, 68·00; H, 5·37; $(OCH_3)_3$, 30·97%.) On mixing with *nor*geodin A trimethyl ether the m.p. was depressed to 187–195°. It is quite insoluble in HCl and forms no ferrichloride.

(*b*) *With diazomethane.* *Nor*geodin A (0·4 g., m.p. 300°) was treated with an excess of ethereal diazomethane. After keeping overnight the insoluble dimethyl ether was filtered off (0·37 g.) and crystallized from ethanol. Colourless, hair-like needles were formed, m.p. 273–275° which gave an intense green colour with $FeCl_3$. (Found: C, 67·61, 67·47; H, 5·22, 5·22; OCH_3, 22·2, 21·9. $C_{16}H_{14}O_5$ requires C, 67·16; H, 4·92; $(OCH_3)_2$, 21·65%.)

*Nor*geodin B, treated similarly, gave a mixture of di- and trimethyl ethers. (Found: OCH_3, 23·3%.)

Attempted resolution of erdin

d-α-Phenyl-ethylamine was obtained by combining the *dl*-base with *l*-malic acid and crystallizing three times from water (cf. Loeven, 1905). The homogeneous product was decomposed with alkali, extracted with ether which was then evaporated to leave the base. To 0·12 g. of the base was added 2 ml. ether and then 0·48 g. erdin (1·5 mol.) in 30 ml. ether. The yellow crystalline precipitate was filtered off (0·27 g.) and crystallized twice from hot water to give yellow needles (33 mg.), m.p. 159–164° decomp. By grinding with 2N-HCl and repeatedly washing with water, the erdin was freed from the base. From dioxane/light petroleum it formed clusters of nitrogen-free pale buff prisms, m.p. 217–220° (decomp.). A 4·82% solution in dioxane showed no rotation in a 0·5 dm. tube. A similar attempt, using brucine, also failed.

Geodin oxime

Geodin (1 g.), hydroxylamine hydrochloride (0·4 g.) and sodium acetate (anhydrous, 1 g.) were refluxed in ethanol (60 ml.), during 45 min. After cooling, a red-brown solid separated which was filtered off. From ether/light petroleum it formed red crystals, m.p. 259–261°; it contained no nitrogen. On acidifying the filtrate with dilute HCl the oxime separated as a pale yellow solid (0·21 g.). From chloroform/light petroleum it formed short yellow needles, m.p. 285–286° (decomp.), and was appreciably soluble in water. An 0·57% solution in chloroform showed no optical activity in an 0·5 dm. tube. (Found: C, 49·0, 49·1; H, 3·3, 3·2; N, 3·9, 3·9; Cl, 17·4. $C_{17}H_{13}O_7Cl_2N$ requires C, 49·3;

H, 3·2; N, 3·4; Cl, 17·15%.) When dihydrogeodin was treated with hydroxylamine hydrochloride under identical conditions it was recovered unchanged, and the crude product contained no nitrogen.

SUMMARY

1. Further degradative studies of geodin and erdin, two chlorine-containing metabolic products of *Aspergillus terreus* Thom, are described leading to definitive structures for the methylation product obtained with dimethyl sulphate and alkali; and for geodin and erdin hydrates, obtained by the action of 80% sulphuric acid.

2. On the basis of this and other information possible structural formulae for the two metabolic products are presented, and their limitations discussed, the chief difficulties being the point of attachment of a second ether link between the rings in a highly substituted *o*-benzoylbenzoic acid, and the apparent lack of an asymmetric centre in erdin.

3. The optical activity of geodin probably lies in a *pseudo*-ester group derived from this structure, while the non-chlorinated ring in each substance has semi-quinonoid rather than phenolic properties.

The authors offer their best thanks to Prof. A. R. Todd, F.R.S., and Dr C. H. Hassall for invaluable criticism and advice, and to Prof. F. L. Bates, Physics Department, University College, Nottingham, for the use of a spectrophotometer.

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Appendix. Antibacterial Activities of Geodin and Erdin

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Methods

A sterile clear 0·4% solution of geodin was prepared by dissolving geodin (40 mg.) in 0·1 N-NaOH (2·01 ml.) and diluting with sterile 2% glucose broth medium (8 ml.). It was used immediately for making the higher dilutions in glucose broth (for tests with all strains of bacteria save one) and in plain heart broth (for test with *Vibrio cholerae* only). For every test, ten serial dilutions were prepared (see Table 1), and the tubes were immediately inoculated with one loopful of a 24 hr. culture of the desired micro-organism. Growth readings were usually taken after 24 hr. incubation at 37°, but the period was 48 hr. for strains of *Corynebacterium diphtheriae* and 65 hr. for mycobacteria. Further tests with mycobacteria were also carried out using nutrient agar instead of liquid glucose broth. In the case of erdin dilutions were made from a 1% solution prepared similarly.

Results

Geodin had no appreciable effect, at a concentration of 0·1% upon the following nine strains of Gram-negative bacteria: *Escherichia coli* (two strains); *Bacterium dysenteriae* Flexner; *Bact. dysenteriae* Sonne; *Salmonella paratyphi* B; *Salm. typhimurium*; *Salm. enteritidis*; *Proteus vulgaris*, and upon one Gram-positive organism (*Mycobacterium phlei*, N.C.T.C. No. 525). It had an appreciable inhibitory effect upon the growth of nineteen Gram-positive strains (including two non-pathogenic strains of mycobacteria) and upon two Gram-negative organisms (*V. cholerae* and *Bact. dysenteriae* Shiga), at limiting dilutions varying from 1 : 1000 to 1 : 128,000 (see Table 1). Erdin had not the slightest inhibitory effect upon any of the above micro-organisms when present at a concentration of 0·1%.

SUMMARY

Geodin may be regarded as a moderately potent antibiotic substance active in the main against Gram-positive bacteria only, while erdin has not been observed to inhibit the growth of bacteria.

Table 1. *Bacteriostatic power of geodin against nineteen Gram-positive and two Gram-negative strains in glucose broth*

Micro-organism	Catalogue No. (National Collection of Type Cultures) *or* London School of Hygiene and Tropical Medicine	Limiting dilution for complete inhibition of growth
Bacterium dysenteriae Shiga	N.C.T.C. 4837	1,000
Vibrio cholerae	N.C.T.C. 1633	2,000 (in plain broth)
Mycobacterium smegmatis	N.C.T.C. 523	2,000
Staphylococcus aureus	N.C.T.C. 4736	4,000
Staph. aureus	N.C.T.C. 3093	8,000
Staph. albus	N.C.T.C. 3256	8,000
Streptococcus viridans	N.C.T.C. 3166	8,000
Myco. smegmatis	N.C.T.C. 523	8,000 (in nutrient agar)
Staph. aureus	N.C.T.C. 4163	16,000
Mycobacterium sp. (saprophytic)	N.C.T.C. 510	16,000
Staph. aureus	N.C.T.C. 3750	32,000
Bacillus subtilis	L.S.H.	32,000
B. anthracis	L.S.H.	32,000
Corynebacterium diphtheriae gravis	L.S.H.	32,000
C. diphtheriae intermedius	L.S.H.	32,000
Staph. aureus	L.S.H.	64,000
Staph. aureus	N.C.T.C. 3761	64,000
Staph. aureus	N.C.T.C. 3095	64,000
C. diphtheriae mitis	L.S.H.	64,000
Staph. citreus	N.C.T.C. 2301	128,000
Strep. pyogenes Griffith type I	N.C.T.C. 2432	128,000
B. anthracis	N.C.T.C. 5444	128,000

Studies on Antibiotics*

2. BACTERIOLOGICAL ACTIVITY AND POSSIBLE MODE OF ACTION OF CERTAIN NON-NITROGENOUS NATURAL AND SYNTHETIC ANTIBIOTICS

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In our search for new antimicrobial agents of natural origin, Dr R. Klein, in our laboratories, found some time ago that the crude culture fluid of *Aspergillus terreus*, strain N.C.T.C. 3911 (Ac 100), possessed a far higher antibiotic activity than could be accounted for by its known content of citrinin (Raistrick & Smith, 1935; 1936). We succeeded in isolating in addition to the latter substance a crystalline, faintly yellow compound which had marked antibiotic activity; this compound was examined chemically by Mr C. H. Hassall and Prof. A. R. Todd of Cambridge University who identified it as geodin, $C_{17}H_{12}O_7Cl_2$, previously isolated from another organism by Raistrick & Smith (1936).

While still engaged on the elucidation of the structure of clavatin or patulin (Bergel, Morrison, Moss & Rinderknecht, 1944; cf. also Raistrick, 1943) we also synthesized a number of compounds possessing some of the structural features of penicillic acid (Birkinshaw, Oxford & Raistrick, 1936) and of the clavatin molecule, which we knew at the time to be an unsaturated keto lactone.

In the following we present the preliminary results of the bacteriological tests and experimental evidence for a possible mode of action of these substances. Numbers following compounds refer to Table 2, where formulae are given.

* Part I, see *J. chem. Soc.* (1944), p. 415.

METHODS AND EXPERIMENTAL

Natural compounds (mycological investigations by R. Klein)

Penicillic acid and clavatin (nos. 1 and 2) were prepared as described by Birkinshaw *et al.* (1936) and Bergel, Morrison, Moss, Klein, Rindernecht & Ward (1943) respectively. For the preparation of geodin (no. 3) from *Aspergillus*

terreus N.C.T.C. 3911 (L.S.H.T.M. Ac 100) cultural conditions were similar to those given by Raistrick & Smith (1935), with the following differences. Stock cultures were kept on Czapek-Dox agar with 1·5% glucose and 2% agar; when these slopes were about 8–14 days old, the spores of one tube were suspended in 10 ml. sterile water. This suspension was used for the inoculation of two 1 l. Erlenmeyer flasks, each containing 50 ml. Czapek-Dox agar with 1·5% glucose and 1% agar. One Erlenmeyer flask culture was used for the inoculation of *c.* fifty 40 oz. square bottles when about 8–14 days old, each bottle containing *c.* 300 ml. Czapek-Dox solution as used by Raistrick & Smith (1935). A spore suspension was made by adding aseptically 250 ml. sterile water to the Erlenmeyer flask and by shaking vigorously for a few minutes. Of this suspension, *c.* 5 ml. were transferred aseptically to each bottle. The bottles were placed on the flat side and kept at 26–27° for 18–20 days. During this time folded mycelial mats with cinnamon spores and yellow transpiration drops were formed on the surfaces of the medium. Samples for activity assays of the culture liquid were taken at regular intervals, and when a satisfactory activity was reached, the liquid was harvested by draining it off the mats.

3 l. of culture fluid were adjusted to pH 6·6 and stirred vigorously with 31 g. of activated charcoal for ½ hr. The charcoal was then filtered off, dried in a desiccator and extracted with three portions of 400 ml. of methanol. The methanol extract was evaporated to a small volume, from which crude geodin crystallized out. Yield: 0·7 g., m.p. 212–213°. It was recrystallized from methanol or aqueous acetone, m.p. 227–230°. (Found: C, 51·2; H, 3·2. Calc. for $C_{17}H_{12}O_7Cl_2$: C, 51·1; H, 3·0%.)

Synthetic compounds

Ethyl Δ^{δ}-α:γ-*diketo*-δ-*methylhexenoate* (no. 4) (prepared by A. R. Moss). Sodium (3·56 g.) was dissolved in 100 ml. ethanol and the excess solvent removed by distillation under reduced pressure. The last traces of ethanol were removed by heating in an oil bath at 180°. Nitrogen was then admitted to the white dry sodium ethoxide and a solution of 22·6 g. of dimethyl oxalate in 130 ml. dry ether was added with ice cooling. Methyl *iso*propenyl ketone (13 g.) was then added over 10 min. and the reaction mixture was allowed to stand overnight. After addition of 100 ml. of dry ether, the yellow sodium derivative of ethyl Δ^{δ}-α:γ-diketo-δ-methylhexenoate was filtered off and kept in a desiccator over P_2O_5. The free ester was obtained by acidification of an aqueous solution of the sodium derivative and extraction with ether. After washing, drying and evaporation of the extract, a mobile oil was obtained which boiled at 71°/0·05 mm. and partly polymerized on distillation. The copper derivative containing 1 molecule of water of crystallization melted at 120–121°, and the anhydrous material at 131–133°, after recrystallization from benzene/light petroleum (b.p. 60–80°). (Found: C, 50·0; H, 4·9. $(C_9H_{11}O_4)_2Cu$ requires C, 50·2; H, 5·1%.)

The sodium enolate of ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate (no. 5) (ethyl α-mesityloxido oxalate) was prepared according to Claisen (1896) (cf. also Borsche & Thiele, 1923).

The sodium enolate of ethylacetylpyruvate (no. 6) and that of ethyl α:γ-diketo-δ-dimethylhexanoate (no. 7) were made according to Claisen & Stylos (1887) and Couturier (1910) respectively.

Ethyl benzoylpyruvate (no. 8) and the corresponding *p-chloro compound* (no. 9), m.p. 65° (found: Cl, 13·7. $C_{12}H_{11}O_4Cl$ requires Cl, 13·8%), were prepared according to Beyer & Claisen (1887).

α-Keto-β-benzoyl-γ-phenylbutyrolactone (no. 10) was prepared according to Knoevenagel (1894).

α-*Keto*-β-*benzoyl*-γ-*methylbutyrolactone* (no. 11). A mixture of ethyl benzoylpyruvate (2·2 g.), paraldehyde (0·45 g.), and benzene (1 ml.) was cooled and saturated with HCl. After standing for 2 days at room temperature the mixture, which had partly solidified, was filtered. The crude reaction product was recrystallized from benzene/light petroleum (b.p. 60–80°). It melted at 130–132° and was a colourless substance soluble in sodium carbonate solution. (Found: C, 66·0; H, 4·9. $C_{12}H_{10}O_4$ requires C, 66·1; H, 4·6%.)

The sodium salt of 5-phenylpyrazole-3-carboxylic acid (no. 12), a derivative of benzoylpyruvic acid, was made according to Bülow (1904).

Δ^{δ}-α:γ-*Diketo*-δ-*methylhexenoic acid* (no. 13) (prepared by A. R. Moss). The ester (no. 4) (21·55 g.) was added to a solution of 5·12 g. of sodium in 35 ml. of ethanol and 60 ml. of water cooled in ice. The mixture was then allowed to stand at room temperature for ½ hr. A cold solution of 7·5 ml. conc. H_2SO_4 in 120 ml. of water was then added. The solid which separated was filtered and washed with a small quantity of ice-water. Crude acid thus obtained was dissolved in carbon tetrachloride from which it crystallized on cooling as a pale yellow substance, melting at 104–107°. Sublimation at 60–70°/0·1 mm. of a sample yielded colourless crystals of m.p. 106–108°. (Found: C, 54·0; H, 5·1. $C_7H_8O_4$ requires C, 53·8; H, 5·1%.)

Chloralide of α:γ-*diketo*-δ:δ-*dimethylhexenoic acid* (no. 14). Ethyl α:γ-diketo-δ:δ-dimethylhexenoate (no. 7) (2 g.) and chloral (4·05 g.) were heated in a sealed tube at 120° for 3 hr. The product thus obtained was triturated with water. The residual oil was distilled at 14 mm. pressure. Some unchanged ethyl α:γ-diketo-δ:δ-dimethylhexenoate was recovered. The solid residue in the distillation flask was recrystallized from benzene/light petroleum (60–80°). The colourless material melted at 156–158°. (Found: C, 40·3; H, 4·3; Cl, 35·2. $C_{10}H_{11}O_4Cl_3$ requires C, 40·0; H, 3·7; Cl, 35·2%.)

The chloralide of benzoylpyruvic acid (no. 15) and the corresponding *p-chloro compound* (no. 16), m.p. 185–187°. (Found: Cl, 39·6. $C_{12}H_6O_4Cl_4$ requires Cl, 39·9%) were prepared according to Schiff (1898).

Benzoylacrylic acid (no. 17) was synthesized according to von Pechmann (1882).

p-Chlorobenzoylacrylic acid (no. 18) (cf. Kohler & Woodward, 1936). Aluminium chloride (56 g.) was added in portions to a mixture of 19·6 g. of maleic anhydride, 22·4 g. of chlorobenzene and 50 ml. of carbon disulphide. The mixture was gradually heated to the boiling-point and refluxed for 3 hr. The solvent was decanted and the residue decomposed with ice and HCl. The crude *p*-chlorobenzoylacrylic acid thus obtained was recrystallized from benzene. The lemon-yellow crystals had m.p. 157–158°. (Found: Cl, 16·6. $C_{10}H_7O_3Cl$ requires Cl, 16·7%.)

2:4-*Dichlorobenzoylacrylic acid* (no. 19). Powdered aluminium chloride (56 g.) was added in portions to a mixture of 19·6 g. of maleic anhydride, 30 g. of 1:3-dichlorobenzene and 50 ml. of carbon disulphide. After refluxing

for 6 hr., the reaction mixture was decomposed with ice and HCl. The mixture was then extracted with ether. The ether was washed with water, dried and evaporated. The residue was triturated with benzene. 2:4-Dichlorobenzoylacrylic acid crystallized from the benzene extract on concentrating. Recrystallization from chloroform gave a yellow crystalline material of m.p. 191–193°. (Found: Cl, 29·0. $C_{10}H_6O_3Cl_2$ requires Cl, 29·0%.)

The enol lactone of 6:6-dimethyl-3-benzoyltetrahydro-γ-pyrone-2-carboxylic acid (no. 20) was prepared from ethyl α-mesityloxidooxalate according to Meyer (1913). Its constitution will be discussed later.

Anhydro-6-methyl-3-hydroxybenzylidene tetrahydropyrone-2-carboxylic acid (no. 21). (*a*) *Ethyl* Δ^{δ}-α:γ-*diketoheptenoate.* Sodium ethoxide (0·68 g.) was suspended in dry ether (10 ml.). After cooling to −15°, ethyl oxalate (1·48 g.) was added and then ethylidene acetone (0·84 g.) with continuous stirring. After standing for 2 days at room temperature, the sodium derivative was filtered and dried over P_2O_5. The copper salt which was prepared by treating the aqueous solution of the sodium derivative with copper acetate melted at 156–160° (decomp.) after recrystallization from light petroleum (b.p. 100–120°). (Found: C, 50·3; H, 5·3. $(C_9H_{11}O_4)_2Cu$ requires C, 50·2; H, 5·1%.)

(*b*) *No.* 21. The enol lactone of 6-methyl-3-benzoyltetrahydro-γ-pyrone-2-carboxylic acid was obtained by shaking an ethereal solution of its copper derivative with dilute ice-cold H_2SO_4, washing, drying and evaporating the ether. This material (1·3 g.) was mixed with 1·0 g. of benzaldehyde and two drops of piperidine. After standing for several weeks the reaction mixture had solidified. The crystalline material was recrystallized from benzene/light petroleum (b.p. 60–80°) and subsequently from methanol. The colourless product melted at 176–177°. (Found: C, 68·6; H, 5·0. $C_{14}H_{12}O_4$ requires C, 68·8; H, 4·9%.)

Bacteriological examination

Geodin (Table 1) was prepared as a 1/300 ethanolic solution with a trace of dilute ammonia. All other substances were tested in aqueous solutions. Serial dilutions were set up in 2% glucose-broth (pH 6·9). One loop of a 1/100 dilution of a 24 hr. culture was used as inoculum; one loop of undiluted culture was used for *Mycobacterium smegmatis.* Incubation was carried out at 37° and growth was observed after 18 hr. and, in the case of geodin, also after 2 days.

RESULTS AND DISCUSSION

Table 1 gives the results of the preliminary bacteriological investigation on geodin. It is interesting to note that in contrast to clavatin and penicillic acid, geodin possesses relatively little activity against Gram-negative organisms and may thus be classed with some of our synthetic inhibitors. Table 2 contains bacteriological data on our synthetic substances and results of our investigation on the mechanism of action of these and of some natural antibiotics.

In our synthetic approach to this problem we chose penicillic acid (no. 1) as a model and first prepared ethyl Δ^{δ}-α:γ-diketo-δ-methylhexenoate (no. 4) which shows a structural resemblance to the latter and was found to possess bacteriostatic activity when tested as the sodium salt of the enolic form. Replacing the *iso*propenyl radicle in no. 4 by other groups, we found that in order to produce substances with antibiotic activity the δ-carbon atom must not carry a hydrogen atom. Thus the sodium enolates of ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate (no. 5); and ethyl α:γ-diketovalerate (ethyl acetylpyruvate; no. 6) were inactive while those of ethyl α:γ-diketo-δ:δ-dimethylhexanoate (no. 7) and ethyl α:γ-diketo-γ-phenylbutyrate (ethyl benzoylpyruvate; no. 8) were slightly active. On the other hand, in compounds such as α-keto-β-benzoyl-γ-phenylbutyrolactone (no. 10) and α-keto-β-benzoyl-γ-methylbutyrolactone (no. 11), where, in contrast to no. 1 and no. 4, both the α- and β-carbon atoms are fully substituted, no activity was found. No. 11 was prepared by condensation of benzoylpyruvic ester with acetaldehyde. Incorporation of the α:β double bond into a heterocyclic ring structure as in no. 12 likewise led to complete loss of activity. The third structural feature which appears to be essential for antibiotic activity is the presence of an α:β double bond as achieved in no. 1 by etherification of the β-enol and in no. 4 by the formation of a sodium derivative of the α-enol, the corresponding free diketo acid (no. 13) being inactive. When the α-enol structure was stabilized in another manner—namely by condensation with chloral as in the formation of β:β:β-trichloroethylidene ether ester of α-hydroxy-β-benzoylacrylic acid (no. 15) and β:β:β-dichloroethylidene ether ester of α-hydroxy-γ-keto-δ:δ-dimethylhexanoic acid (no. 14)—a striking increase in activity was observed amounting in no. 15 to about three times that of clavatin, as tested against *Staph. aureus.*

Table 1. *Effect of geodin on bacterial growth*

Organism tested	Highest dilution of geodin giving complete inhibition of growth for 18 hr.	2 days
Staphylococcus aureus N.C.T.C.* 4163	1 : 2,000,000	1 : 1,000,000
Staph. aureus N.C.T.C. 4163 (in 10% serum)	1 : 80,000	1 : 10,000
Bacillus subtilis N.C.T.C. 85	1 : 1,000,000	1 : 1,000,000
Streptococcus pyogenes (Richards) (in 10% blood)	1 : 40,000	—
Pseudomonas fluorescens	1 : 20,000	1 : 10,000
Esch. coli N.C.T.C. 86	1 : 40,000	1 : 20,000
Mycobacterium smegmatis	1 : 20,000	1 : 10,000

* National Collection of Type Cultures.

Table 2. *Compounds tested, and their effects on bacterial growth in the presence and absence of cysteine*

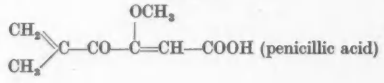
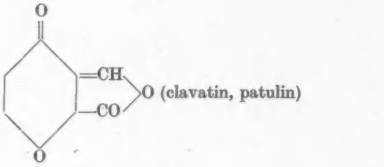
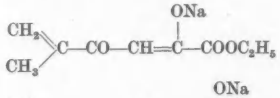
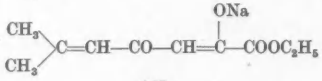
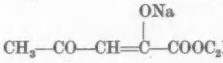
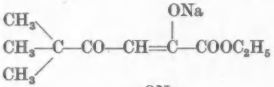
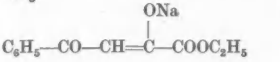
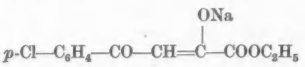
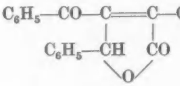
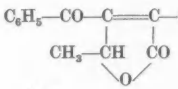
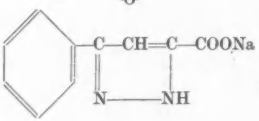
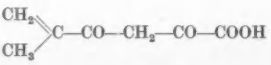
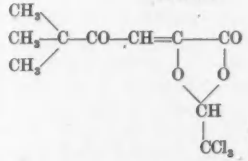
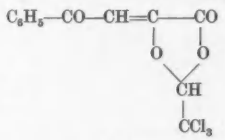
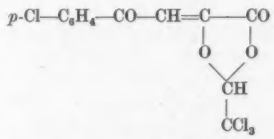
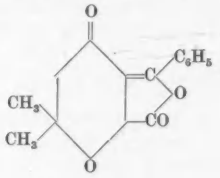
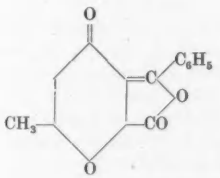
Compound		Highest dilution giving complete inhibition of growth			
		Without cysteine		With cysteine (1 : 1000, buffered at pH 6·8)	
No. (see text)	Formula	*Staph. aureus* N.C.T.C. 4163	*Esch. coli* N.C.T.C. 86	*Staph. aureus* N.C.T.C. 4163	*Esch. coli* N.C.T.C. 86
1		1 : 16,000	1 : 25,000	1 : 1,000	1 : 1,000
2		1 : 100,000	1 : 200,000	<1 : 1,000	1 : 1,000
3	$C_{17}H_{12}O_7Cl_2$ (geodin)*	1 : 2,000,000	1 : 30,000	<1 : 32,000	<1 : 8,000
4		1 : 5,000	—	—	—
5		<1 : 1,000	—	—	—
6		<1 : 1,000	—	—	—
7		1 : 2,000	—	1 : 2,000	—
8		1 : 4,000	—	1 : 4,000	—
9		1 : 2,000	—	1 : 1,000	—
10		<1 : 1,000	—	—	—
11		<1 : 1,000	—	—	—
12		<1 : 100	—	—	—
13		1 : 1,000	—	—	—

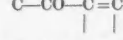
Table 2 (*cont.*)

No. (see text)	Compound: Formula	Highest dilution giving complete inhibition of growth: Without cysteine — *Staph. aureus* N.C.T.C. 4163	Without cysteine — *Esch. coli* N.C.T.C. 86	With cysteine (1 : 1000, buffered at pH 6·8) — *Staph. aureus* N.C.T.C. 4163	With cysteine (1 : 1000, buffered at pH 6·8) — *Esch. coli* N.C.T.C. 86
14		1: 24,000	—	—	—
15		1 : 300,000	<1 : 30,000	<1 : 30,000	<1 : 30,000
16		1 : 300,000	—	—	—
17	C_6H_5—CO—CH=CH—COOH	1 : 25,000	1 : 15,000	<1 : 1,000	<1 : 3,000
18	*p*-Cl—C_6H_4—CO—CH=CH—COOH	1 : 50,000	1 : 5,000	1 : 1,000	<1 : 1,000
19	2 : 4-Cl_2—C_6H_3—CO—CH=CH—COOH	1 : 80,000	1 : 5,000	1 : 1,000	<1 : 1,000
20		<1 : 1,000	<1 : 1,000	—	—
21		1 : 1,000	—	—	—

* On the constitution of geodin see Calam, Clutterbuck, Oxford & Raistrick (1947).

Finally when the enolic double bond in α:β-position was replaced by a simple ethylenic linkage, as in benzoylacrylic acid, substances of relatively high antibacterial activity were obtained (cf. nos. 17, 18, 19). The benzoylacrylic acids were obtained by condensation of maleic anhydride with the appropriate benzenes.

Summarizing, the following structure:



possessing a hydrogen atom either on the α or β carbon atom, may be regarded as being responsible for antibacterial activity. The same features are

present in the clavatin molecule (no. 2) with the exception of a complete substitution of the carbon atom corresponding to the δ-position of the above formula. It is noteworthy that here again (cf. nos. 10 and 11) lack of hydrogen on both carbon atoms of the double bond adjacent to the keto group leads to loss of activity, as observed with compounds nos. 20 and 21 and with *iso*clavatin (Puetzer, Nield & Barry, 1945).

The former substances were prepared by condensation of benzaldehyde with the enolates of ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate and ethyl Δ^{δ}-α:γ-diketoheptenoate respectively. No. 20 has been erroneously described by Meyer (1913) as the lactone of γ-hydroxybenzyl-mesityloxidooxalic acid. However, its chemical behaviour which follows that of the cyclic form of β-mesityloxidooxalate very closely and recent work by Puetzer *et al.* (1945), on similar substances, leaves little room for doubt on the structure assigned to it in Table 2.

Effects of cysteine

Some years ago we found independently of Geiger & Conn (1945) that the antibacterial activities of clavatin (no. 2) and benzoylacrylic acid (no. 17) were inhibited by the presence of cysteine. We have subsequently confirmed cysteine-inactivation of penicillic acid and observed the same phenomenon with our synthetic compounds, nos. 15, 18 and 19, but not with the sodium enolates nos. 7, 8 and 9, which are all derivatives of pyruvic acid and have relatively low initial activities. It is noteworthy that geodin as well as our synthetic substances which are inactivated by cysteine possess comparatively low activity against *Esch. coli*, in contrast to the natural antibiotics clavatin and penicillic acid.

Geiger & Conn (1945) suggested that the mechanism of the antibiotic action of clavatin and penicillic acid may be an interaction of their α:β-unsaturated keto group with the sulphydryl group of an enzyme system vital for bacterial metabolism. They associate the order of antibiotic activity of the compounds included in their investigation with their efficacy in adding sulphydryl compounds like cysteine and thioglycollic acid. Our observations on the synthetic compounds, nos. 15, 16, 17, 18 and 19, support this hypothesis for α:β-unsaturated ketones possessing the essential features indicated in a preceding paragraph. It is significant, however, that the increase in antibiotic activity of our compound, no. 17 (benzoylacrylic acid), against Gram-positive organisms which follows the introduction of chlorine into its benzene nucleus is not accompanied by a parallel increase in activity against Gram-negative bacteria. This together with the fact that, in contrast to clavatin and penicillic acid, none of the synthetic substances and geodin possess activity against Gram-negative bacteria of the same order as that against Gram-positive organisms, indicates that some additional structural feature is involved in the antibacterial mechanism of clavatin and penicillic acid which is absent in the synthetic compounds as well as geodin (cf. Calam, Clutterbuck, Oxford & Raistrick, 1947). Cavallito & Haskell (1945) have obtained evidence that this may be the unsaturated lactone structure which is present in clavatin and potentially present in penicillic acid. The results of these authors and those obtained in this paper permit the tentative conclusion that the inhibition of Gram-negative organisms by α:β-unsaturated ketones of the type specified above involves an enzyme system containing SH groups different from that in Gram-positive bacteria, or that the enzyme system in Gram-negative bacteria is less accessible to the action of these compounds than is the case for Gram-positive organisms.

The mode of action of our substances, nos. 4, 7, 8 and 9 which are derivatives of pyruvic acid and are not inhibited by cysteine, seems to be entirely different.

SUMMARY

1. Geodin has been isolated from the culture medium of *Aspergillus terreus* N.C.T.C. 3911 and shown to possess antibiotic activity of a high order against Gram-positive bacteria.

2. The synthesis of certain substances chemically related to penicillic acid and clavatin are described and some features essential for antibiotic activity are pointed out.

3. The inhibitory effect of cysteine on these antibiotics has been investigated and its possible significance in relationship to the mode of action of these substances is discussed.

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The Importance of Folic Acid and Unidentified Members of the Vitamin B Complex in the Nutrition of Certain Insects

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In our work on the nutrition of insects evidence has been accumulating that growth on purified diets containing all the known vitamins of the B complex in pure substance is inferior to that on similar diets which contain yeast or yeast extracts. This was particularly noticeable in the genus *Ephestia* (Lepidoptera) where the caterpillars always grew much more slowly in the absence of yeast or yeast fractions (Fraenkel & Blewett, 1946*a*). From the effect of adding fractions resulting from a charcoal treatment of yeast extract, it seemed probable that the missing factor might be 'folic acid' and this was finally established by tests with synthetic folic acid (Lederle). An even more striking case of the need for folic acid was found in the mealworm, *Tenebrio molitor*, the growth rate of which on certain diets was vastly increased in the presence of folic acid. In our earlier work with another beetle, *Tribolium confusum*, it seemed that a mixture of all the then known vitamins of the B complex would sustain growth equally as well as yeast or a yeast extract (Fraenkel & Blewett, 1943*b*). This result has been difficult to repeat on several occasions but it has now been established that a folic acid deficiency can also be demonstrated in *Tribolium* if the casein in the diet is sufficiently purified.

'Folic acid' is used throughout this paper to signify an unspecified preparation showing folic acid activity, while folic acid designates synthetic folic acid (Lederle) (pteroylglutamate, Angier, Boothe, Hutchings, Movat, Semb, Stokstad, Subbarow, Waller, Gosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith, 1946), which was formerly called *L. casei* factor. Some of the results have been briefly reported elsewhere (Fraenkel & Blewett, 1946*b*).

METHODS

The methods of breeding and of testing and preparing diets have been fully described (Fraenkel & Blewett, 1943*a*, *b*, 1946*a*). All tests were performed at 25° and 70% relative humidity. As a rule, 20 larvae were used in each test. All tests were started with newly hatched larvae and the criterion for the efficiency of a diet was either the length of the larval period (*Ephestia kuehniella*, *E. elutella*, *Tribolium confusum*), or the time taken until emergence of the adult (*Plodia interpunctella*), or growth expressed by the weight increase (*Tenebrio molitor*).

In most experiments the effect of the following three diets was compared:

(1) *Pure vitamins diet.* Casein (Glaxo Laboratories) 20, glucose anhydr. (British Drug Houses Ltd.) 80, McCollum's salt mixture (no. 185) 2, 'insoluble yeast' (yeast exhaustively extracted by boiling water) 2·5, cholesterol 1 and water 10 parts. To this mixture the following vitamins were added (expressed as μg./g. of dry diet): aneurin 25, riboflavin 12, nicotinic acid 25, pyridoxin 12, panthothenic acid 25, choline chloride 500, *i*-inositol 250. In tests with *Tribolium*, the diet consisted of 50 parts of casein and 50 parts of glucose. In tests with *Ephestia* and *Plodia*, 1 part of wheat-germ oil (Vitamins Ltd.) was added.

(2) *Yeast diet.* No. 1 with the addition of 1% dried brewer's yeast (Glaxo Laboratories).

(3) *Folic acid diet.* No. 1 with the addition of synthetic folic acid (Lederle).

The following preparations were also used:

(1) Yeast extract and yeast charcoal filtrate and eluate. Yeast extracts prepared from fresh brewers' yeast according to Chick & Roscoe (1930) or from dried brewers' yeast (Glaxo Laboratories) by a similar method, were added to the diets in quantities corresponding to 5% yeast. The charcoal treatment of yeast was carried out according to Hutchings, Bohonos & Peterson (1941) (adsorption at pH 3 with three successive portions of norite and elution of the norite adsorbates with ammoniacal ethanol).

(2) Grass juice (spray dried, Cerophyll Laboratories Inc.) was used in 10% solution in hot water. Charcoal treatment was carried out as described for yeast extract.

(3) A liver charcoal filtrate (El Sadr, Macrae & Work, 1940) originally prepared as a riboflavin-free source of B vitamins, and found to be free from folic acid.

(4) Synthetic folic acid (Lederle Laboratories), crystalline vitamin B_c (Parke, Davis and Co.), synthetic xanthopterin (Organon Laboratories).

Yeast and grass juice extracts were added to the diets in quantities which included half the normal quantity of water, and the pure vitamins were dissolved in the other half.

All the graphs concerned with *Ephestia*, *Plodia* or *Tribolium* show the total number of pupae or adults formed plotted against time. The results of tests with *Tenebrio* are expressed in weight curves.

RESULTS

Ephestia kuehniella, E. elutella, Plodia interpunctella

The caterpillars of these moths always grow much more slowly on diets which contain pure vitamins than in the presence of yeast or yeast extracts. This

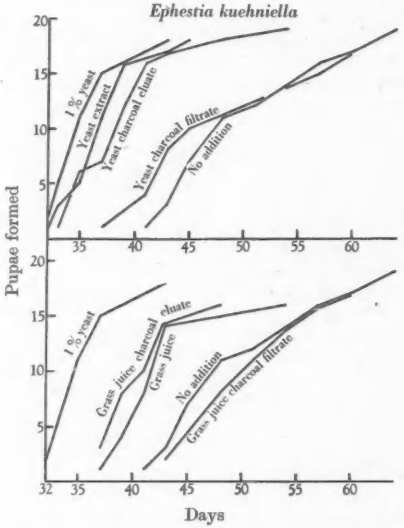

Fig. 1. Growth of *Ephestia kuehniella* on the pure vitamins diet with the addition of yeast, yeast extract, grass juice or the charcoal filtrate or eluate from yeast extract or grass juice.

is illustrated in Figs. 1–4 and also by Fraenkel & Blewett (1946*a*, Figs. 6–9). In a first attempt to identify the missing factor the effect of a preparation of grass juice (Cerophyll Laboratories Inc.) was investigated and it was found that 1% of this added to the pure vitamins diet had a pronounced positive effect on the rate of growth (Fig. 1). This grass juice had been described as a rich source of 'folic acid'. An attempt was therefore made to fractionate grass juice and yeast extract by a charcoal treatment which consisted essentially of the first steps in the concentration of 'folic acid', as described by Hutchings *et al.* (1941).

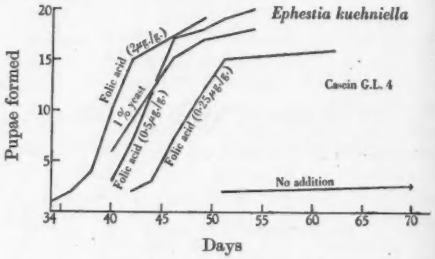

Fig. 2. Growth of *Ephestia kuehniella* on the pure vitamins diet with the addition of graded doses of synthetic folic acid.

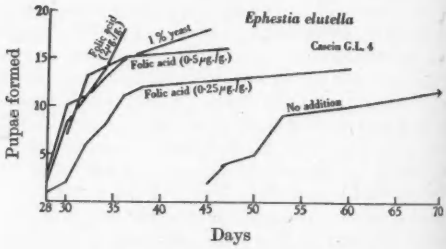

Fig. 3. Growth of *Ephestia elutella* on the pure vitamins diet with the addition of graded doses of synthetic folic acid.

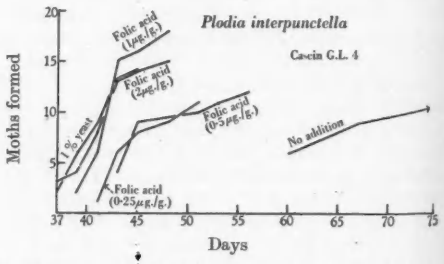

Fig. 4. Growth of *Plodia interpunctella* on the pure vitamins diet with the addition of graded doses of synthetic folic acid.

The results obtained with grass juice and yeast fractions were identical (see Fig. 1). Growth was greatly improved in the presence of the norite-eluate fractions while the filtrate fractions had little effect. This suggested that the factor which was missing in the pure vitamins diet might be 'folic acid'.

Highly potent concentrates of vitamin B_c (Parke, Davis and Co.) were later tested and found to have a positive effect on growth. Finally synthetic folic acid (Lederle Laboratories) was found to be as effective as yeast or yeast extract for *Ephestia kuehniella*, *E. elutella* and *Plodia interpunctella*. Figs. 2–4 give the result of tests with graded doses of folic acid on the development of these species. In each case growth was very slow in the absence of folic acid and the mortality high. With optimal quantities of folic acid growth was as good as on the control diets which contained 1% yeast. The minimum optimal quantity of folic acid for the two *Ephestia* species appeared to be 0·5 μg./g. and for *Plodia* 1 μg./g. of the dry diet.

Crystalline vitamin B_c (Parke, Davis and Co.) seemed to have an even better effect than synthetic folic acid (Lederle). Growth was optimal with as little as 0·25 μg./g., which was the smallest quantity tested (Table 1).

Table 1. *The effect of vitamin B_c and xanthopterin on the growth of* Ephestia kuehniella

Addition to diet	Larval period (days) Range	Average	No. of pupae (out of 20)
No addition	109–121	112·5	4
1% yeast	35–73	58·3	12
Vitamin B_c 0·25 μg./g.	37–54	49·3	14
Vitamin B_c 0·5 μg./g.	37–60	52·2	15
Vitamin B_c 1·0 μg./g.	38–63	52·6	16
Vitamin B_c 2·0 μg./g.	37–54	47·5	13
Xanthopterin 10 μg./g.	57–76	68·4	5
Xanthopterin 100 μg./g.	44–120	98·0	8
Xanthopterin 1000 μg./g.	43–76	55·7	16

Table 2. *The effect of folic acid, liver charcoal filtrate and xanthopterin on the growth of* Ephestia kuehniella

Addition to diet	Larval period (days) Range	Average	No. of pupae (out of 20)
No addition	61–95	78·4	5
1% yeast	44–61	50·9	17
Folic acid 2 μg./g.	38–54	44·0	16
Folic acid 2 μg./g. + liver extract	38–52	42·6	17
Xanthopterin 10 μg./g.	—	88	1
Xanthopterin 100 μg./g.	61–84	76·3	3
Xanthopterin 1000 μg./g.	50–95	64·7	15

Folic acid can be replaced by xanthopterin, but only when very large quantities of the latter are used. Even 1000 μg./g. of xanthopterin did not seem as effective as 2 μg./g. of synthetic folic acid or 0·25 μg. of B_c (Tables 1, 2). A positive effect with xanthopterin was obtained on two occasions, but not on a third.

Finally, the question arose whether any unspecified B vitamins were required by *Ephestia kuehniella* in addition to folic acid and the other known B vitamins. Addition to a folic acid diet of a liver charcoal filtrate which had proved to be essential for the development of *Tenebrio* had no further effect on the rate of growth of *Ephestia* (Table 2).

Tenebrio molitor

In early tests with the meal worm, *Tenebrio molitor*, on the effect of charcoal filtrates and eluates from yeast extract, the results were very different from those already described for *Ephestia kuehniella*. Growth was poor in the presence of the eluate, and very much improved in the presence of the filtrate, though still short of growth on diets which contained yeast or unfractionated yeast extract (Fig. 5).

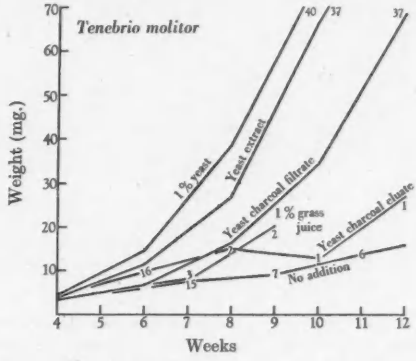

Fig. 5. Growth of *Tenebrio molitor* on the pure vitamins diet with the addition of yeast, yeast extract, a charcoal filtrate or eluate from yeast extract, or grass juice. The numbers on the curves indicate the numbers alive, out of 40 larvae.

Addition of a grass juice, which had a positive effect on *Ephestia* (Fig. 1) had none on *Tenebrio* (Fig. 5). A striking feature of these tests was the fact that the larvae died off quickly on diets which contained no addition, and still more quickly in the presence of the yeast eluate or grass juice, while they survived in the presence of yeast, yeast extract or yeast filtrate. From these results it was at first concluded that *Tenebrio* did not require folic acid, a conclusion which proved entirely erroneous.

In a subsequent test, yeast charcoal filtrate was replaced by a liver charcoal filtrate which had been treated with charcoal for the purpose of removing riboflavin (El Sadr *et al.* 1940) and which, from the nature of this treatment could also be expected to be free from 'folic acid' (Hutchings *et al.* 1941). Very little growth took place in the presence of the liver charcoal filtrate or of synthetic folic acid alone, but in the presence of both growth was as fast as with 1% yeast (Fig. 6).

The results of this test demonstrate that besides folic acid, *Tenebrio* also requires an unknown factor which is contained in the charcoal filtrate of yeast or

liver extracts. Without this factor and without folic acid, growth is slow and the larvae die after 4–6 weeks. Without this survival factor, the presence of folic acid, either in pure substance or in a charcoal eluate or in grass juice, has the effect of increasing both the rate of growth and the mortality.

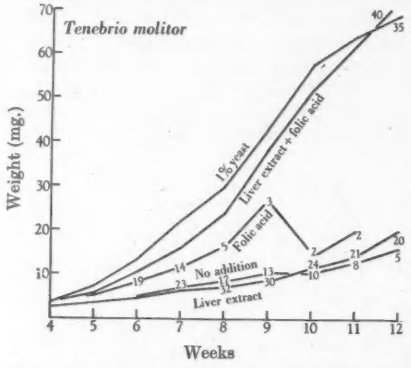

Fig. 6. Growth of *Tenebrio molitor* on the pure vitamins diet with the addition of yeast, liver charcoal filtrate and/or synthetic folic acid. The numbers on the curves indicate the numbers alive, out of 40 larvae.

It still remains to explain why on the former occasion to which Fig. 5 refers a yeast charcoal filtrate had such a good effect on growth in the absence of the eluate which, by analogy with the tests with *Ephestia*, would be expected to contain the bulk of the folic acid. The following explanations offer themselves: (1) *Tenebrio* might require significantly less folic acid than *Ephestia*. (2) The casein, which was used on that former occasion (Fig. 5) might have contained significant amounts of folic acid. (3) The fractionation of the yeast extract might not have been perfect and significant amounts of folic acid might have remained in the charcoal filtrate.

(1) In a test on the folic acid requirements of *Tenebrio* it was found that 0·125 μg./g. gave an optimal response, against 0·5 μg./g. with *Ephestia*, i.e. an amount four times smaller (Table 3).

(2) This test on the folic acid requirements was carried out simultaneously with four different preparations of casein, three prepared by Glaxo Laboratories (G.L.) and one an American preparation (Labco). In the absence of folic acid and the presence of the liver charcoal filtrate by far the best growth was obtained on casein G.L. 3 which is the same as that used on the occasion to which Fig. 5 refers, when good growth occurred in the absence of the yeast charcoal eluate. Growth on casein G.L. 4 in the presence of liver charcoal filtrate was intermediate between G.L. 3 and 6 and was still inferior on Labco casein. In the presence of sufficient quantities of synthetic folic acid and liver charcoal filtrate growth was as fast as with 1 % yeast. There was also no significant difference between the three G.L. caseins on these diets, but on Labco casein growth was significantly slower. These results suggest that G.L. 3 casein contains more folic acid than the other casein preparations and that Labco casein contains very little, if any. Similar growth effects on these caseins were also obtained with another insect, *Tribolium confusum* (Fig. 7) on diets which contained no additional folic acid.

(3) The results of Table 3 show clearly that the liver charcoal filtrate must have been virtually free from folic acid, and exactly the same result has been more recently obtained with a yeast charcoal filtrate.

Further evidence is given in Table 3 to the effect that folic acid in the absence of liver extract has a pronounced effect on growth, while on most occasions only about 10 % of the larvae were alive after 10 weeks. Liver extract in the absence of folic acid always had a striking effect on survival, which however, was never as good as in the presence of additional folic acid except with casein G.L. 3 which, as

Table 3. *Growth and survival of* Tenebrio molitor *on artificial diets containing four different preparations of casein, and with the addition of graded doses of folic acid and/or liver charcoal filtrate*

(40 larvae were used in each test. Survival after 10 weeks is expressed as a percentage of the number of larvae which were alive after 5 weeks.)

	Casein G.L. 3		Casein G.L. 4		Casein G.L. 6		Labco casein	
Addition to diet	Average weight (mg.)	Survival (%)	Average weight (mg.)	Survival (%)	Average weight (mg.)	Survival (%)	Average weight (mg.)	Survival (%)
No addition	7·0	4·1	8·9	33·3	7·1	33·3	6·3	13·6
Folic acid (2 μg./g.)	55·5	10·0	29·5	9·0	34·2	33·3	50·0	12·5
Liver charcoal filtrate (l.c.f.)	31·8	88·8	15·0	69·2	8·4	66·6	5·4	38·4
l.c.f. + folic acid (0·06 μg./g.)	—	—	38·2	100	31·7	100	23·3	100
l.c.f. + folic acid (0·12 μg./g.)	—	—	59·5	72·2	44·9	96·1	43·6	100
l.c.f. + folic acid (0·25 μg./g.)	—	—	50·6	100	51·1	100	43·3	95·6
l.c.f. + folic acid (0·5 μg./g.)	—	—	54·2	84·6	46·2	100	34·6	100
l.c.f. + folic acid (1·0 μg./g.)	—	—	54·8	100	45·0	100	43·1	100
l.c.f. + folic acid (2·0 μg./g.)	50·3	100	53·1	100	45·5	81·8	36·2	96·4
1 % yeast	54·3	95·5	56·5	100	53·8	100	41·3	100

pointed out above, must have contained significant amounts of folic acid; survival was lowest on Labco casein which, as already suggested from the weight figures, must have contained significantly less folic acid (if any) than caseins G.L. 3 and 4.

In tests with *Tenebrio*, folic acid could not be replaced by xanthopterin, even in quantities up to 8000 times larger than effective quantities of folic acid (Table 4).

Table 4. *Growth and survival of* Tenebrio molitor *on the pure vitamins diet with the addition of liver charcoal filtrate and xanthopterin or folic acid.*

(40 larvae were used in each test. Survival after 10 weeks is expressed as a percentage of the number of larvae which were alive after 5 weeks.)

Addition to diet	Average weight (mg.)	Survival (%)
No addition	8·9	33·3
Folic acid (2 μg./g.)	29·5	9·0
Liver charcoal filtrate (l.c.f.)	15·0	69·2
l.c.f. + xanthopterin (10 μg./g.)	11·7	77·2
l.c.f. + xanthopterin (100 μg./g.)	12·7	72·7
l.c.f. + xanthopterin (1000 μg./g.)	14·6	80·0
l.c.f. + folic acid (2 μg./g.)	53·1	100

Tribolium confusum

In our earlier work the conclusion was reached that the larvae of the flour beetle *Tribolium confusum* grew as well on a synthetic diet which contained all the then known B vitamins as the controls which received 5% yeast in their diet (Fraenkel & Blewett, 1943*b*). From this it appeared that *Tribolium* did not require additional unknown B factors. This result has not been confirmed on later occasions. In comparing the results of such diets it became clear that the most obvious variable factor was the casein of which a number of different samples had been obtained in the course of time. This was in fact borne out in a test in which the effect of the pure vitamins diet, described on p. 469, was tested simultaneously with six different samples of casein. Of these caseins, five were obtained between 1939 and 1945 from Glaxo Laboratories, and one from British Drug Houses, and all the samples were of 'vitamin and fat free' quality. The result, given in Fig. 7, showed that the efficiency of these diets differed very widely. With three of the caseins, G.L. 2, G.L. 3 and B.D.H., growth on the pure vitamins diet was as good or nearly as good as on the control diet with 5% yeast. G.L. 4 gave very much slower growth. The most striking difference between the efficiency of a yeast and a pure vitamins diet was obtained on a later occasion, when Labco casein was used (Fig. 8). Addition of synthetic folic acid to a pure vitamins diet, with either G.L. 4 or Labco casein made these diets as efficient as the yeast diet (Fig. 8) and exactly the same result was obtained with another species, *T. castaneum*, on a G.L. 4 casein diet. From this we may conclude that the differences in the efficiency of the diets revealed in Figs. 7 and 8 is due to the differences in folic acid

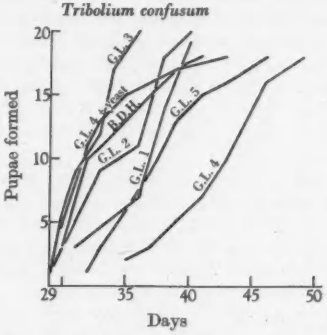

Fig. 7. Growth of *Tribolium confusum* on the pure vitamins diet using six different casein preparations. G.L. = Glaxo Laboratories; B.D.H. = British Drug Houses.

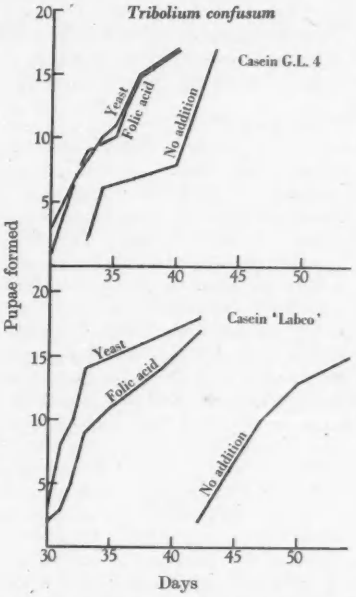

Fig. 8. Growth of *Tribolium confusum* on the pure vitamins diet with the addition of yeast or synthetic folic acid. G.L. = Glaxo Laboratories.

content of the caseins. G.L. 3 apparently contains the most folic acid, Labco the least and G.L. 4 an intermediate quantity as was concluded from the experiments with *Tenebrio*, already described.

The minimum optimal requirement for folic acid of *T. confusum* was found to be 0·125–0·25 μg./g. of

the dry diet. This result agrees well with the quantity of 0·15 μg./g. calculated by Grob, Reichstein & Rosenthal (1945) from tests with a very impure concentrate of folic acid. Cannon, Boutwell & Elvehjem (1945) have reported that the folic acid content of various casein preparations may vary between 0·19 and 0·03 μg./g. according to the casein and test organism used. The higher of these figures is of an order which ensures optimal growth of *Tribolium* and we have shown that this does occur on certain casein preparations.

From the results shown in Fig. 8 it appears that artificial diets which contain all the known B vitamins, including folic acid, are as good for *Tribolium* as control diets with 5 % yeast. This is not quite correct. In all the cases so far mentioned 2·5 % of the diets consisted of the water insoluble fraction of yeast which serves as a source of biotin. When 'insoluble yeast' is replaced by biotin, growth is noticeably delayed. The biotin diet becomes optimal with the addition of 1 % yeast, but is not improved by the addition of yeast extract which suggests that the missing factor is not easily soluble in water. The need of *Tribolium* for an unknown food factor, which is contained in the water-insoluble fraction of yeast has also been pointed out recently by Grob *et al.* (1945).

DISCUSSION

From the results presented it is clear that folic acid is an important growth factor for the six insects so far investigated, viz. *Tribolium confusum*, *T. castaneum*, *Ephestia kuehniella*, *E. elutella*, *Plodia interpunctella* and *Tenebrio molitor*. The minimum optimal quantities required were very similar in all cases: 0·125–0·25 μg./g. dry diet for *Tribolium*, 0·5 μg./g. for the two *Ephestia* species and 0·125 μg./g. for *Tenebrio*. For *Ephestia kuehniella*, vitamin B_c appeared even more efficient than folic acid, but it would be dangerous to draw conclusions from a single test, especially as crystalline vitamin B_c is now considered to be identical with synthetic folic acid. These quantities are very similar to those reported for the chick and stated by Luckey, Moore, Elvehjem & Hart (1946*a*) as 0·25–0·5 μg./g., and by Hutchings, Oleson & Stokstad (1946) as 0·5–1·0 μg./g. We have so far determined minimum optimal quantities of the chemically known vitamins for one insect only, *Tribolium confusum* (unpublished), and obtained the following values: thiamin 1 μg./g. diet, riboflavin 1–2 μg./g., nicotinic acid 8 μg./g., pyridoxin 1·0 μg./g., pantothenic acid 4 μg./g. and biotin 0·05 μg./g. Folic acid has therefore an optimal effect in quantities significantly smaller than any of the B vitamins except biotin.

From the evidence so far obtained by us it appears that, for *Ephestia kuehniella* and *Tribolium confusum*, a diet which contains all the known factors of the vitamin B complex, including folic acid and the water insoluble fraction of yeast, is not improved by the addition of liver or yeast extracts. *Tenebrio molitor*, on the other hand, fails to develop on diets which contain all the known members of the B complex, including folic acid and the water insoluble fraction of yeast. The missing substance, to which we shall in future refer as vitamin B_T (because it is necessary for *Tenebrio*) is contained in a charcoal filtrate prepared from yeast or liver extract. Similar results, showing the importance of at least another factor in liver or yeast, in addition to folic acid, have recently been reported for the guinea-pig (Woolley & Sprince, 1944), the mink (Schaeffer, Whitehair & Elvehjem, 1946), a monkey (Cooperman, Elvehjem, McCall & Ruegamer, 1946), and the chick (Luckey, Moor, Elvehjem & Hart, 1946*a*, *b* and Petering, Marvel, Glausier & Waddell, 1946) and similar conclusions are reached in the latest reports about the effect of folic acid in the treatment of certain nutritional anaemias (Wills, 1946; Watson & Castle, 1946). The possibility is not excluded that the missing factors reported in experiments with such widely different material may in the end prove identical. The characterization of the charcoal filtrate factor which is essential for the survival of *Tenebrio* will be the subject of a subsequent paper.

SUMMARY

1. Synthetic folic acid (Lederle) is an important growth factor for six insect species. In the absence of folic acid, growth is very slow (*Tribolium*, *Ephestia*) or ceases entirely (*Tenebrio*), and the mortality is high (*Ephestia*, *Tenebrio*).

2. For *Ephestia kuehniella*, crystalline vitamin B_c has an effect as good as, if not better than, synthetic folic acid.

3. Xanthopterin has a similar effect on *Ephestia*, but only in quantities approximately 1000 times larger than those used of folic acid. It has no effect on *Tenebrio*.

4. The minimum optimal requirements for folic acid of the insects investigated are between 0·125 and 0·5 μg./g. of dry diet.

5. *Tenebrio* requires in addition to folic acid and the other known B vitamins a factor, contained in a charcoal filtrate prepared from liver or yeast extract, which we have named vitamin B_T.

We gratefully acknowledge a grant from the Medical Research Council for the study of insect nutrition of which this investigation is a part. We are also indebted to Parke, Davis and Co. (Detroit) for gifts of vitamin B_c concentrates and crystalline vitamin B_c, to Lederle Laboratories (Pearl River, N.Y.) for gifts of synthetic folic acid, to Cerophyll Laboratories (Kansas City, Mo.) for gifts of spray dried grass juice, and to Dr A. M. Copping (Lister Institute) and to Glaxo Laboratories for liver charcoal filtrates and to Dr W. C. J. Ross (Organon Laboratories) for a gift of synthetic xanthopterin.

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Linoleic Acid and Arachidonic Acid in the Metabolism of Two Insects, *Ephestia kuehniella* (Lep.) and *Tenebrio molitor* (Col.)

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In a foregoing paper (Fraenkel & Blewett, 1946*b*) we have shown that the larvae of the moth *Ephestia kuehniella* and of two other *Ephestia* species grow badly on artificial diets in the absence of wheat-germ oil and that the effect of wheat-germ oil could be attributed principally to two substances: (1) linoleic acid, which is necessary for emergence and normal formation of the scales on the wings, and (2) vitamin E which is necessary for rapid growth and as an antioxidant for linoleic acid. The effect of linolenic acid was found to be similar to that of linoleic acid. Arachidonic acid had not been investigated at the time and we shall show in the present paper that unlike linoleic acid it has no positive effect on the scales and emergence. In our former paper we also showed that other antioxidants, viz. gallates and ascorbic acid can be substituted for vitamin E and we shall now give more data concerning the effect of ascorbic acid. Finally, we have now compared the linoleic acid contents of *Ephestia* larvae, grown in the presence and absence of linoleic acid, by observing the effects of their oils on the development of *Ephestia*, and similarly of the mealworm, *Tenebrio molitor*, which does not require linoleic acid in the diet. It has thus been established that synthesis of linoleic, or possibly linolenic, acid takes place in *Tenebrio* but not in *Ephestia*.

METHODS

The methods of preparing the diets, breeding and testing have already been fully described (Fraenkel & Blewett, 1946*a*, *b*). The fat-free diet used for *Ephestia* and *Tenebrio* consisted of

Casein (extracted, Glaxo Laboratories)	20 parts
Glucose (anhydrous)	80 parts
Yeast (dried brewer's, debittered, Glaxo Laboratories)	5 parts
Cholesterol	1 part
McCollum's salt mixture no. 185	2 parts
Water	10 parts

RESULTS

The effect of arachidonic acid

The tests with arachidonic acid were carried out in two quantities, 5 mg. and 1·7 mg./g. of the dry diet, and in combination with either α-tocopherol (1 mg./g.) or the unsaponifiable fraction of wheat-germ oil (5 mg./g.). There was no positive effect whatsoever on scales or emergence in any of the tests with arachidonic acid but growth was always accelerated in its presence (Fig. 1). This was especially striking in combination with vitamin E, which by itself had a lesser growth-promoting effect than the unsaponifiable fraction of wheat-germ oil. It is

very improbable that the absence of the specific 'linoleic acid activity' of arachidonic acid was due to its becoming ineffective by oxidation during the course of the tests. In that case we would have expected toxic effects to become apparent, as has been the case in certain tests with linoleic acid (Fraenkel & Blewett, 1946*b*). It is also improbable

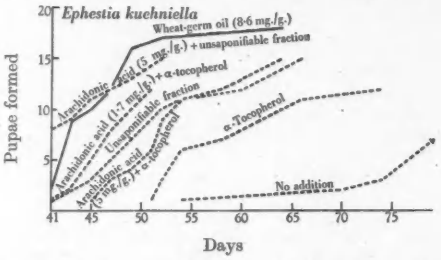

Fig. 1. The effect of arachidonic acid, α-tocopherol and the unsaponifiable fraction of wheat-germ oil on the growth of *Ephestia kuehniella*. —— Normal moths emerge; - - - no moths emerge.

that arachidonic acid would have shown a positive effect on the scales if it had been used in larger quantities. Linoleic acid produced an optimal effect with 4 mg./g. and a very noticeable effect with as little as 0·3 mg./g. There is a striking similarity between the effects of arachidonic acid and the highly unsaturated docosahexaenoic acid from cod-liver oil, which similarly did not improve the scale condition or emergence but accelerated growth very considerably (Fraenkel & Blewett, 1946*b*).

It is worth noting that α-tocopherol by itself on this occasion very considerably improved the rate of growth.

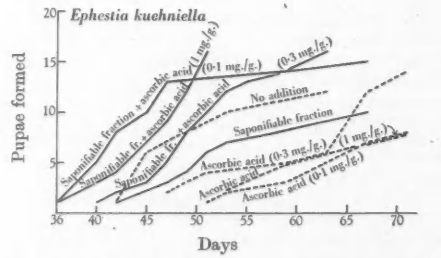

Fig. 2. The effect of ascorbic acid and the saponifiable fraction of wheat-germ oil on the growth and emergence of *Ephestia kuehniella*. —— Normal moths emerge; - - - no moths emerge.

The effect of ascorbic acid

We have formerly shown (Fraenkel & Blewett, 1946*b*) that the effects of α-tocopherol, i.e. promoting growth and stabilizing linoleic acid, can to some extent be reproduced by other antioxidants, e.g. gallates, and there were indications that ascorbic acid had a similar action. On repeating tests with ascorbic acid we have now obtained a very striking acceleration of growth in its presence. It is evident from Fig. 2 that ascorbic acid does not act by itself but only in combination with fatty acids from which we may conclude that this effect is principally that of an antioxidant. Too great quantities of ascorbic acid are toxic. Addition of ascorbic acid alone, without the saponifiable fraction of wheat-germ oil, also has a toxic effect.

Synthesis of linoleic acid by insects

Judging from the importance of linoleic acid in the diet of *Ephestia*, one would expect that this species does not synthesize it, unless to an entirely insufficient degree. Insects which develop normally in the absence of linoleic acid may either not require it or synthesize it in sufficient quantity. To obtain evidence of the synthesis of linoleic acid, body fat from *Ephestia* and *Tenebrio*, grown in the presence and absence of linoleic acid, was tested for linoleic acid activity, using *Ephestia* itself as the test subject. Four different samples of oil were prepared, by extraction with light petroleum (Soxhlet), from *Ephestia* and *Tenebrio* larvae, grown on the following diets:

(1) *Ephestia* diet with linoleic acid: whole meal flour.

(2) *Ephestia* diet without linoleic acid: the artificial diet described above with the addition of 1% cod-liver oil, which as already shown by us (Fraenkel & Blewett, 1946*b*) has an excellent effect on growth and no effect on scales.

(3) *Tenebrio* diet with linoleic acid: whole meal flour with the addition of 10% yeast.

(4) *Tenebrio* diet without linoleic acid: as no. 2 but using ether-extracted yeast, and without cod-liver oil.

To record the states of linoleic acid deficiency which become apparent on certain diets, a classification of deficiencies has been used which is principally based on the appearance of scales on the wings. In this classification, which is fully explained elsewhere (Fraenkel & Blewett, 1946*b*), class 1 signifies a normal moth, class 8 failure to emerge from the pupa, while 2–7 indicate increasing tendency of the wings to lose their scales. The effect of the various insect oils described above on the degree of linoleic acid deficiency is shown in Table 1.

The effects of the oils from *Ephestia kuehniella* and *Tenebrio* grown on a diet with linoleic acid are similar. 9·3 mg./g. of *Tenebrio* oil supplies just sufficient linoleic acid for perfect wings and the same quantity of *Ephestia* oil is almost sufficient. With smaller quantities of oils the wings become progressively deficient in scales. With the oils from

insects grown on a diet without linoleic acid the difference between the two insects is very striking. Even with the largest quantities of *Ephestia* oil, the scale condition is about the same as in a diet which contains the unsaponifiable fraction of wheat-germ oil alone. The corresponding *Tenebrio* oil used as 9·3 mg./g. has an effect as good as if not better than 4·6 mg./g. *Tenebrio* oil from a linoleic acid-containing diet. From this we may conclude that hardly any synthesis of linoleic acid occurs in *Ephestia*, while there is considerable synthesis in *Tenebrio*.

grown on different diets by comparing their 'linoleic acid' effect with that of graded doses of wheat-germ oil, as determined in our former publication. The minimum optimal quantity was then found to be 4·3 mg. wheat-germ oil, containing about 55 % of linoleic plus a small percentage of linolenic acid. The condition with 2·3 mg. wheat-germ oil corresponded roughly to the effect of 9·3 mg. *Ephestia* oil grown in the presence of linoleic acid and the effect of the corresponding *Tenebrio* oil was somewhat better. This would suggest a 'linoleic acid' content of the

Table 1. *The effect of various oils on the degree of linoleic acid deficiency in* Ephestia kuehniella

Additions to diet (per g.)		Classes of deficiencies (Fraenkel & Blewett, 1946*b*) 1	2	3	4	5	6	7	8	Total pupae from 20 larvae	Average pupation time (days)
No addition		—	—	—	—	—	—	—	11	11	77·8
Wheat-germ oil 8·6 mg.		18	—	—	—	—	—	—	—	18	46·9
Unsaponifiable fraction of wheat-germ oil		—	—	—	—	—	—	5	10	15	52·3
Fat from *Tenebrio* grown with linoleic acid	1·15 mg.	—	—	—	1	3	4	4	3	15	52·3
	2·3 mg.	—	2	3	3	3	6	2	—	19	54·8
	4·6 mg.	—	2	5	5	3	1	1	1	18	54·0
	9·3 mg.	12	2	—	—	—	—	1	—	15	48·6
Fat from *Tenebrio* grown without linoleic acid	1·15 mg.	—	—	—	1	1	2	1	9	14	61·7
	2·3 mg.	—	—	—	1	2	4	3	3	13	57·5
	4·6 mg.	—	—	1	6	3	4	4	—	18	55·0
	9·3 mg.	—	4	4	2	1	—	3	—	14	45·2
Fat from *Ephestia* grown with linoleic acid	1·15 mg.	—	—	—	—	2	4	1	8	15	54·1
	2·3 mg.	—	—	1	—	3	7	4	3	18	52·2
	4·6 mg.	—	2	4	5	3	1	2	—	17	48·5
	9·3 mg.	5	7	3	—	—	—	—	—	16*	48·6
Fat from *Ephestia* grown without linoleic acid	1·15 mg.	—	—	—	—	—	1	—	16	17	59·9
	2·3 mg.	—	—	—	—	—	1	2	15	18	60·6
	4·6 mg.	—	—	—	—	—	—	5	9	14	51·4
	9·3 mg.	—	—	—	—	—	2	3	10	15	53·1

* 1 damaged.

This difference in the linoleic acid content of oils from *Ephestia* and *Tenebrio* grown on a diet without linoleic acid is the more striking in view of the fact that the diet of *Ephestia* may have contained very small quantities of linoleic acid in the dried yeast, while the yeast in the *Tenebrio* diet was ether extracted.

In all tests, addition of the insect oils to the diet had a marked positive effect on the growth rate, which was of the same order as that induced by wheat-germ oil (Table 1, last column). This effect was most noticeable in the diets with the largest amount of oils. It had little relation to the linoleic acid content of the oils and was still marked with the smallest quantities of oil used. Oil from *Ephestia* grown in the absence of linoleic acid had a nearly maximum effect on growth and a minimum effect on the scales. In this it closely resembles cod-liver and halibut-liver oil (Fraenkel & Blewett, 1946*b*).

An attempt has been made to estimate the amounts of linoleic acid in the oils of our two insects *Ephestia* oil of about 15 %, and of the *Tenebrio* oil of possibly 20 %. The effect of the *Tenebrio* oil, grown in the absence of linoleic acid, was about half that of the oil grown in the presence of linoleic acid. The corresponding *Ephestia* oil showed a minute linoleic acid activity, suggesting a content of less than 1 %; too small to be measured with any degree of accuracy.

These results do not answer directly the question whether linoleic acid is of importance in the metabolism of such insects as do not require it as a constituent of their food, but its formation in appreciable quantities in the bodies of insects of the latter class would suggest that it served some particular purpose in their metabolism.

SUMMARY

1. Arachidonic acid, unlike linoleic acid, has no positive effect on the development of the wing of *Ephestia*, but it has a positive effect on growth of the

larva. In both respects it resembles the effect of docosahexaenoic acid from cod-liver oil.

2. Ascorbic acid, in combination with the saponifiable fraction of wheat-germ oil, has a positive growth effect which is attributed to an antioxidant action.

3. The linoleic acid content of various insect oils has been estimated by testing their effect on the wings of *Ephestia*. Oils from *Tenebrio* and *Ephestia* grown on a diet containing linoleic acid were assessed to contain 20 and 15% linoleic acid, and from a diet without linoleic acid, 10 and less than 1% respectively. It is concluded that linoleic acid is synthesized by *Tenebrio* but not by *Ephestia*.

We gratefully acknowledge a grant by the Medical Research Council for the study of insect nutrition, of which this investigation is a part. We are also indebted to Prof. G. O. Burr and Dr R. T. Holman (Minneapolis) for a sample of methyl arachidonate.

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Further Observations on the Non-fermentability of *d*-Fructopyranose by Yeast

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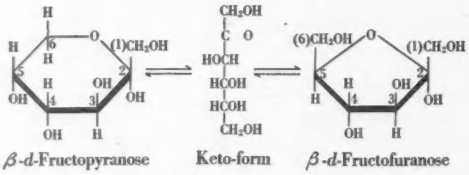
In recent years we have investigated the fermentation rates of the various *d*-fructose, *d*-glucose and *d*-mannose modifications at low concentration (0·005 M) under conditions (0°, pH 4·5) which reduce the rate of mutarotation to a minimum. On solution in water β-*d*-fructopyranose is reversibly converted to β-*d*-fructofuranose, the keto form contributing only a minute proportion to the equilibrium mixture



(Purves & Hudson, 1934; Isbell & Pigman, 1938; Bednarczyk & Marchlewski, 1938). The proportion of fructofuranose increases with temperature. The mutarotation of *d*-glucose, on the other hand, consists almost exclusively in an α-pyranose ⇌ β-pyranose change.

The results obtained by this technique (for review, see Gottschalk, 1947*b*) seemed to substantiate the hypothesis, first proposed by Hopkins (1931), that only the furanose form of *d*-fructose is fermentable by yeast, whereas the pyranose modification is unfermentable. This interpretation implies that the concentration of *d*-fructofuranose, and not the total fructose concentration, has to be considered when the fermentation rate is referred to substrate concentration.

As an alternative interpretation of the slower fermentation rate, as compared with *d*-glucose, of *d*-fructose in low concentration at 30° and at 0° (cf. nos. 1 and 3 of the summary at the end of section 'Discussion') the following explanation may be offered: A lower concentration of *d*-fructose inside the cell resulting in a slower fermentation rate may be due to a higher energy barrier encountered by *d*-fructopyranose at the interface suspending fluid-cell membrane. Thus it was recently shown (Gottschalk, 1947*a*) that at 10° *d*-mannopyranose in low concentration (0·026 M) is fermented at only half the rate of *d*-glucose by living yeast, whereas equal rates of CO_2 production are observed with yeast extract as fermenting agent under otherwise similar conditions. In view of this observation it seemed desirable to test the correctness of the alternative interpretation of the abnormal behaviour of yeast towards *d*-fructose in low concentration. If, by resisting the penetration of *d*-fructose more than that of *d*-glucose, the membrane of the yeast cell causes the slower fermentation of low concentrations of fructose compared with glucose, then the fermentation rates both of *d*-fructose and *d*-glucose may be expected to vary in the same way with yeast concentration. The present paper is concerned with the effect of yeast concentration on the fermentation rate of *d*-fructose and of *d*-glucose at low concentrations at 25°.

MATERIALS AND METHODS

The experiments were performed with baker's yeast (Effront), stored for 24 hr. at 1–2° after having been compressed. The yeast (2·0 g.) was suspended in 0·1 M-KH_2PO_4

(total vol. 40 ml.; initial pH 4·5). From this stock solution a 1:1 dilution was prepared with 0·1 M-KH_2PO_4, and 20 ml. of the required suspension were taken for each experiment.

The substrates added were pure, anhydrous substances: $[\alpha]_D^{20°}$ *d*-glucose: +52·4° (water; $c=4$, $l=2$); $[\alpha]_D^{20°}$ *d*-fructose: −91·7° (water; $c=4$, $l=2$).

The rate of fermentation at 25·0°±0·1 was determined with the apparatus described previously (Gottschalk & Rawlinson, 1942). After thermal and gaseous equilibration of the suspension at 25° with N_2 (freed from O_2) the sugar, dissolved in 0·5 ml. of 0·1 M-KH_2PO_4 and allowed to mutarotate overnight at 25°, was tipped into the yeast suspension. Readings were taken at 2 min. intervals over a period of 20 min.; the values observed were reduced to N.T.P.

RESULTS

It may be seen (Table 1) that on doubling the amount of yeast the maximum rate of CO_2 production from *d*-fructose (0·005 M) increases only by one-third, whereas with glucose the fermentation rate is proportional to the yeast concentration. It may be mentioned that with these concentrations of yeast and substrate 77 % of the hexose consumed is fermented to CO_2 and ethanol.

Table 1. *Fermentation rates of* d-*fructose and* d-*glucose* (0·005 M) *with various yeast concentrations*

Temp. 25°.

Exp. no.	Yeast (g.)	Hexose added (mg.)	CO_2 produced (ml. in successive 2 min. periods) 0–2	2–4	4–6	6–8	8–10	10–12	12–14	Maximum fermentation rate (ml. CO_2/2 min.)
			A. With *d*-fructose as substrate							
1	0·5	19·3	0·24	0·37	0·36	0·36	0·30	0·24	0·24	0·37
	1·0	19·4	0·49	0·48	0·49	0·36	0·30	0·24	0·18	0·49
2	0·5	19·3	0·24	0·36	0·36	0·36	0·36	0·24	0·18	0·36
	1·0	19·3	0·49	0·48	0·48	0·36	0·30	0·17	0·18	0·49
3	0·5	19·5	0·24	0·37	0·36	0·37	0·36	0·24	0·24	0·37
	1·0	19·6	0·48	0·49	0·48	0·42	0·29	0·24	0·24	0·49
4	0·5	18·9	0·24	0·37	0·36	0·36	0·36	0·30	—	0·37
	1·0	19·2	0·42	0·47	0·48	0·47	0·29	0·30	—	0·48
			B. With *d*-glucose as substrate							
5	0·5	19·1	0·37	0·48	0·49	0·54	0·54	0·42	0·18	0·54
	1·0	19·3	0·72	0·96	0·96	0·58	0·18	—	—	0·96
6	0·5	19·7	0·37	0·48	0·49	0·48	0·48	0·48	0·24	0·49
	1·0	19·6	0·73	0·84	0·96	0·60	0·24	—	—	0·96
7	0·5	20·1	0·31	0·41	0·52	0·52	0·48	0·47	0·24	0·52
	1·0	20·0	0·61	0·86	0·97	0·72	0·24	—	—	0·97

DISCUSSION

The finding that at low substrate concentration the fermentation rate of *d*-glucose varies directly with yeast concentration, while that of *d*-fructose does not, disproves the interpretation that differences in the permeability of the cell membrane for the two sugars may be responsible for the peculiar reaction of yeast towards *d*-fructose at low concentrations. The observed effect of yeast concentration on the fermentation rates of *d*-fructose and of *d*-glucose is, however, to be expected if the fermentation of *d*-fructose, in contrast to that of *d*-glucose, is preceded by a reversible chemical reaction which is not controlled by the enzyme system of the yeast:

$$d\text{-fructopyranose} \rightleftharpoons d\text{-fructofuranose} \rightarrow \text{fermentation products.} \quad (1)$$

It is evident from (1) that fermentation interferes with the rate of mutarotation in that it cuts down the back reaction by transforming fructofuranose into CO_2 and ethanol. If yeast is added to an equilibrated solution of *d*-fructose, the preformed fructofuranose will be fermented at a rate proportional to the yeast concentration (cf. Table 1, time interval 0–2 min.). Thereafter no proportionality between fermentation rate and yeast concentration will obtain because the effect on the fermentation rate of any increase in yeast concentration is partly counterbalanced by a lower concentration of *d*-fructofuranose. With higher yeast concentration the concentration of *d*-fructofuranose is lower since initially more of the preformed amount is removed and this removal at a higher rate is continued throughout the process.

In conclusion the differences so far established between *d*-glucose and *d*-fructose as fermentation substrates for yeast may be summarized:

(1) At 30° and at substrate concentrations less than 0·03 M, *d*-fructose (equilibrated) is fermented markedly slower than *d*-glucose, whereas at higher concentrations (above 0·1 M) both sugars are fermented at about equal rates (Hopkins & Roberts, 1935*a*).

(2) Between 0·056 and 0·278 M-substrate concentration the temp. coefficients (from 15 to 40°) of fermentation of

d-glucose and *d*-fructose are the same. With 0·014 M-fructose concentration, however, the temp. coefficients of fermentation are lower than those for fructose at higher concentration or for glucose at equal concentration; they approach closely the corresponding temp. coefficients of the rate of mutarotation of *d*-fructose (Hopkins & Roberts, 1935*b*).

(3) At 0° and at 0·005 M-substrate concentration the fermentation rate of β-*d*-fructopyranose is only a small fraction of that of α-*d*-glucopyranose; it coincides practically with the rate of mutarotation of *d*-fructopyranose at 0° (Gottschalk, 1943*a*). Under the same conditions fructofuranose, set free enzymatically from sucrose or raffinose, is readily fermented (Gottschalk, 1945).

(4) Living yeasts of the industrial type (baker's, brewer's, spirit yeasts), and dried yeasts prepared from them, respond uniformly to invert sugar as substrate by fermenting preferentially *d*-glucose at room temperature (for literature see Gottschalk, 1947*c*). However, by raising the proportion of fructofuranose without altering the total concentration of *d*-fructose and *d*-glucose the relative rates at which *d*-glucose and *d*-fructose are fermented from invert sugar can be equalized (Gottschalk, 1945).

(5) The capacity of Sauternes yeast selectively to ferment fructose from invert sugar is confined to the intact cell. Dried Sauternes yeast preferentially ferments glucose from invert sugar (Gottschalk, 1946).

(6) At 25° and at 0·005 M-substrate concentration increase in yeast concentration does not result in a proportional increase in the rate of fructose fermentation, whereas this is the case with glucose as substrate (present paper). The same holds for experiments performed at 0° (Gottschalk, 1943*a*).

The concept that, of the various *d*-fructose modifications present in a fructose solution at equilibrium, only *d*-fructofuranose (22% of the total at 25°; Gottschalk, 1943*b*) is a suitable substrate for ethanolic fermentation seems to be the only interpretation consistent with the relevant facts. Obviously *d*-fructopyranose is unfermentable, because it has no esterifiable —OH group at C atom 6. The α- and β-pyranose forms of *d*-glucose and *d*-mannose and the furanose form of *d*-fructose have as common features the same side-chain and the same spatial distribution of the addenda to C atoms 3, 4 and 5; therefore they can combine with yeast hexokinase by the same mechanism (Gottschalk, 1947*b*).

SUMMARY

1. At 25° and low substrate concentration (0·005 M) the fermentation rate of *d*-glucose is proportional to the concentration of yeast, whereas that of *d*-fructose is not, increasing only by one-third for a twofold increase in yeast concentration.

2. This phenomenon conforms to the concept that of the various *d*-fructose modifications present in solution only *d*-fructofuranose is a suitable substrate for alcoholic fermentation.

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